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[signature] 7/27/00

PI - Signature Date

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Introduction

This is an Academic Award (Career Development Award). The purpose of this application is to free additional time for the Principal Investigator to *"..appraise critically the state of the science in a particular aspect of breast cancer research and to forge new avenues of investigation."* The PI will apply new, state-of-the-art technologies to identify key endocrine-regulated molecular pathways to apoptosis/proliferation. By identifying key components of these pathways, we may be able to predict response to first-line and crossover antiestrogenic therapies, and/or provide novel therapeutic strategies for antiestrogen resistant tumors. We also will establish a SAGE database containing the molecular profile of MCF-7 cells and several variants.

Body

This is an Academic Award, for which a detailed research plan was not required. Since the award is to support academic development, the aims are not finite, *i.e.,* restricted only to the time frame or resources provided through this type of award. Furthermore, unlike a R01-style application, the amount of work proposed represents the efforts of a number of individuals and funded grants already active within the PI's laboratory, and both ongoing and future collaborations with other laboratories. Consistent with this, the proposed work requires substantially more than the time and financial resources provided by a single R01. Without describing the work in this manner, it was unclear how we could address the requirements of this new award category. The aims, amount of work proposed (which must, *e.g.,* go beyond the three year limit to satisfy the award requirements) and time frames were presented, in the original application, with these issues in mind.

Aim 1: We will expand the MCF7/LCC1 and MCF7/LCC9 databases to a minimum of 30,000 tags/database. We also expect to establish a 30,000 tag database for MCF-7 cells growing with and without 17β-estradiol. Completion of all four databases will require longer than the three year period, since we also plan to perform functional studies on candidate genes identified from our comparisons of the MCF7/LCC9 and MCF7/LCC1 databases. For the purposes of this application's duration, we would consider this aim to have been successfully completed once the MCF7/LCC1 and MCF7/LCC9 databases have each reached a size of 30,000 tags. Time: years 1-3.

We have chosen to primarily focus on completing the initial gene microarray studies. However, the MCF7/LCC1 and MCF7/LCC9 SAGE databases are of a sufficient size to warrant publication. We have approximately 11,000 tags in the MCF7/LCC1 database and approximately 13,000 in the MCF7/LCC9 database (Table 1). We have a draft manuscript describing these data and data from the initial gene microarray studies. We expect to submit the final manuscript within the next month, once the remaining repeats of the functional studies are completed.

Table 1: Characteristics of the SAGE Libraries identified from MCF7/LCC1 and MCF7/LCC9 cells (left) and distribution of gene-hit frequency of Tags by the 7,221 known genes (right).
[1]The number of Tags representing a corresponding number of gene hits, *e.g.*, 5,708 Tags are specific for single genes, whereas 208 Tags could hit up to 3 genes each.
[2]Number of Tags
[3]Includes ESTs

Characteristics of SAGE Libraries		Tags[1]	Gene Hits
Tags sequenced from MCF7/LCC1 cells	12,816[2]	5,783	1
Tags sequenced from MCF7/LCC9 cells	11,109	1,170	2
Number of Tags identified	10,518	208	3
Number of known Tags[3]	7,221	38	4
Number of unknown Tags	3,297	10	5

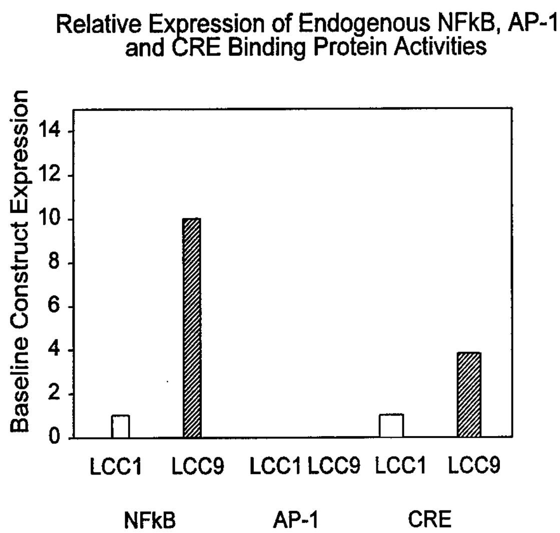

Figure 2: Basal expression of the transcriptional activities of NFκB, CRE and AP-1

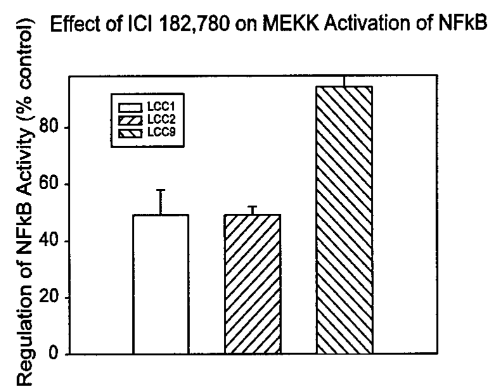

Figure 1: Regulation of NFκB expression in ICI182,780 responsive (LCC1, LCC2) and resistant (LCC9) cells.

Aim 2: We will continue to investigate the functional relevance of those genes/proteins that receive sufficient priority This will include transient transfection studies with promoter-reporter constructs (for transcription modulating factors) and stable transfections to assess functional relevance. We also will investigate clinical relevance by exploring expression in breast tumor biopsies, and correlating expression (or lack thereof) with established prognostic variables, *e.g.,* lymph node status, ER expression, S-phase/proliferation, tumor grade, disease free and overall survival and response to endocrine and cytotoxic chemotherapies. For the purposes of this application's duration, we would consider this aim to have been successfully completed if we can confirm the roles of *NPM*, NFκB, AP-1 and the IRF-1 polymorphism. Time: years 1-3.

We have now identified several candidate genes involved in estrogen and antiestrogen resistance from our 2D-gel, gene microarray and SAGE studies. These include nucleophosmin (*NPM*), interferon regulatory factor-1 (IRF-1), cAMP response element binding activities (CRE) and nuclear factor kappa B (NFκB). Of the transcription factors identified, we have completed promoter-reporter studies with NFκB, AP-1 and CRE. These data demonstrate a 10-fold increase in NFκB activity (Fig 1), and a loss of its regulation by ICI 182,780 (Fig 2), a 4-fold increase in CRE (Fig 1), which is not regulated by ICI 182,780 in either responsive or resistant cells (not shown). We cannot detect AP-1 activity without induction with PKA (Fig 1). PKA-induced AP-1 activity remains antiestrogen regulated in both responsive and resistant cell lines (not shown).

These data suggest that antiestrogen resistant cells can upregulate transcription factors that are not normally regulated by estrogen receptors, *e.g.,* CRE. In addition, some factors that are regulated by estrogen receptors can be upregulated and their estrogenic regulation lost, *e.g.,* NFκB. We are currently investigating the hypothesis that cells have several possible mechanisms available to respond to prolonged antiestrogenic exposures. As described in the original application, these include p53-independent pathways such as NPM/IRF-1, and increased expression and altered regulation of cell survival factors such as NFκB. As indicated above, we are preparing a manuscript describing these initial observations, combining data from both SAGE and gene microarray studies.

Aim 3: We will continue to integrate the emerging experimental data into our molecular transduction schemes, and amend these as appropriate. Clearly, this will require substantial ongoing effort to integrate the studies from the more broad-based projects, *e.g.,* SAGE and gene array, with the more focused and functional studies, *e.g.,* those specifically addressing the function of *NPM* and IRF-1 polymorphism. For the purposes of this application's duration, we would consider this aim to have been

successfully completed once we have established the validity of the *NPM*/YY1/IRF-1 signaling components (p53 independent). Time: years 1-3.

Preliminary data with IRF-1 transfectants suggest that overexpression inhibits the rate of cell proliferation, anchorage-dependent colony formation and estrogen-dependent tumorigenesis in antiestrogen and estrogen responsive MCF-7 cells. These data are consistent with IRF-1 potentially acting as a tumor suppressor gene in breast cancer, and are consistent with our initial hypotheses. These data are currently being repeated, and will be presented in next year's report, since we hope to complete these studies and submit for publication within the next year.

We have recently obtained IRF-1 null mice from Jackson Laboratories, and have generated *NPM* transgenic mice. The phenotypes of these mice, relative to mammary gland development and carcinogenesis, will be explored in the coming year. We also will begin to cross-breed these mice to further evaluate the *NPM*/IRF-1 signaling component described in the original application.

Each of these aims represent ongoing studies within the PI's laboratory and each will continue beyond the limitations of this award. We will continue to evaluate new methodologies and adapt our approaches and integrative studies in the light of published work from other laboratories. ***In this latter regard, the award will specifically allow the PI to spend more time critically appraising the state of science in the area of resistance to estrogens and antiestrogens in breast cancer.***

Key Research Accomplishments (bulleted)

- Completed initial gene microarray and SAGE studies
- Completed analysis of transcriptional activity of initial candidate genes
- Initiated functional studies of IRF-1 and *NPM* (transfections and genetically manipulated mouse modeling)
- Completed and submitted major review on cellular and molecular mechanisms of antiestrogen resistance

Reportable Outcomes

Reportable outcomes are presented as A. Manuscripts, Abstracts and Presentations; B. Other Professional Activities; C. Degrees; and D. Funding Applied for Based on Work Supported by this Award.

A. Manuscripts, Abstracts and Presentations

Consistent with the goals of allowing the PI to spend time reevaluating his field, the PI has recently completed and submitted a major review entitled "Cellular and Molecular Pharmacology of Antiestrogen Action and Resistance". If accepted for publication, a copy will be included in next year's annual report. A copy of the Abstract and Table of Contents is included in the appendix. Other related reviews also have been completed and published/submitted (8,9).

Among the relevant publications supported by this award are data showing the ability of TGFβ to suppress natural killer cell activity as one component of antiestrogen resistance (1). We have identified a new estrogen receptor negative (*de novo* resistant) cell line (2), and investigated the effects of antiestrogens on vascular smooth muscle cell proliferation, since antiestrogens have beneficial effects on the incidence of cardiovascular disease (3). Other studies have implicated protein phosphatase 2A in estrogen-independent growth (4). We have also further investigated the importance of the timing of exposure on the sensitivity of the mammary gland to the development of estrogen-dependent mammary tumors (4-7), and described the use of promoter-reporter assays to investigate estrogen receptor function (10).

Manuscripts

1. Arteaga, C.L., Koli, K.M., Dugger, T.C. **& Clarke, R.** "Reversal of tamoxifen resistance of human breast carcinomas *in vivo* with neutralizing anti-transforming growth factor (TGF)-β antibodies" *J Natl Cancer Inst*, 91: 46-53, 1999.

2. Thompson, E.W., Sung, V., Lavigne, M., Baumann, K., Azumi, N., Aaron, A.D. **& Clarke, R.** "LCC-15-MB: a human breast cancer cell line from a femoral bone metastasis" *Clin Exptl Metastasis,* 17:193-204, 1999.

3. Gopalakrishna, R., Gundimeda, U., Fontana, J.A. **& Clarke, R.** "Differential distribution and nuclear association of protein phosphatase 2A in human breast carcinoma cell lines and its relation to estrogen receptor status and tumor progression." *Cancer Lett* 136:134-151, 1999.

4. Lavigne, M.C., Ramwell, P.W. **& Clarke, R.** "The effects of estrogens and antiestrogens on the growth of porcine coronary artery smooth muscle cells." *Steroids* 64:472-480, 1999.

5. Hilakivi-Clarke, L.A., Onojafe, I., Raygada, M., Cho, E., Skaar, T. **& Clarke, R.** "Prepubertal exposure to zearalenone or genistein reduces mammary tumorigenesis." *Br J Cancer* 80:1682-1688, 1999.

6. Hilakivi-Clarke, L.A., Cho, E., Onojafe, I., Raygada, M. **& Clarke, R.** "Maternal exposure to genistein during pregnancy increases mammary tumorigenesis in female rat offspring." *Oncol Rep* 6:1089-1095, 1999.

7. Hilakivi-Clarke, L.A., Trock, B. **& Clarke, R.** "The estrogenicity of selected nutrients, phytochemicals, pesticides and pollutants: their potential roles in breast cancer." In: *"Breast Cancer, Molecular Genetics, Pathogenesis and Therapeutics.* Series: *Contemporary Cancer Research."* Editor: Bowcock, A. Publisher: Humana Press, Clifton, pp537-568, 1999.

8. **Clarke, R.** & Johnson, M.D. "Chapter 22: Animal models." In: *"Diseases of the Breast"* 2nd Edition. Editors: Harris, J.R., Lippman, M.E., Morrow, M., Hellman, S. Publisher: J. B. Lippincott Co., Philadelphia, pp319-333, 2000.

9. **Clarke, R.,** Leonessa F., Brünner, N. & Thompson, E.W. "Chapter 23: *In vitro* models." In: *"Diseases of the Breast"* 2nd Edition. Editors: Harris, J.R., Lippman, M.E., Morrow, M., Hellman, S. Publisher: J. B. Lippincott Co., Philadelphia, pp335-354, 2000.

10. **Clarke, R.,** Skaar, T., El-Ashry, D., Leonessa, F. & Hilakivi-Clarke, L.A. "Use of ERE and reporter gene constructs to assess putative estrogenic activity." *J Med Food* 2:127-133, 1999.

Abstracts

11. Pu, L.P., Skaar, T.C., Gu, Z.P., Leonessa, F. **& Clarke, R.** "A novel selection system for identifying growth suppressed human breast cells." *Proc Am Assoc Cancer Res* 40: 32, 1999.

12. Lee, R.Y., Skaar, T.C., Leonessa, F. **& Clarke, R.** "The aquisition of retinoid resistance to 4HPR and 9-cis-RA in estrogen independent breast cancer." *Proc Am Assoc Cancer Res* 40: 61, 1999.

13. Gu, Z., Hanfelt, J., Hurley, C., Xaio, Y., Gray, F., Flessate-Harley, D. **& Clarke, R.** "High throughput gene expression profiles associated with antiestrogen responsive *vs.* resistant breast cancer cells." *Proc Am Assoc Cancer Res* 40:158-159, 1999.

14. Skaar, T.C., Bouker, K.B. **& Clarke, R.** "Retinoid regulated genes in breast cancer cells." *Clin Cancer Res*, in press, 1999.

15. Lee, R.Y., Skaar, T.C., Gu, Z., Leonessa, F., **& Clarke, R.** "Retinoid crossresistance to 9-*cis*-RA and 4-HPR is not associated with the loss of RARα and RXRα RNA expression." *Clin Cancer Res*, in press, 1999.

16. Welch, J.N., Chrysogelos, S.A. **& Clarke, R.** "Modulation of epidermal growth factor receptor expression by chemotherapeutic agents in breast cancer cell lines." *Clin Cancer Res*, in press, 1999.

17. Bouker, K.B., Skaar, T.C. **& Clarke, R.** "Interferon regulatory factor-1: a putative mediator of antiestrogen responsiveness in breast cancer." *Clin Cancer Res,* in press, 1999.

18. Gu, Z., Davis, N., Hanfelt, J., Hurley, C., Xiao, H., Gray, F., Flessate-Harley, D. **& Clarke, R.** "Gene expression profiles associated with antiestrogen responsive versus resistant human breast cancer cells." *Clin Cancer Res*, in press, 1999.

19. Bouker, K.B., Skaar, T.C. **& Clarke, R.** "IRF-1 as a mediator of responsiveness to antiestrogens in breast cancer" *Proc Am Assoc Cancer Res*, 41: 427-428, 2000.

20. Skaar, T.C., Bouker, K.B., Barto, T.L., **& Clarke,** R. "Interferon regulatory factor-1 (IRF-1) in breast cancer cells"*Proc Am Assoc Cancer Res*, 41: 428, 2000.

Presentations

1. Research Genetics, Inc. Huntsville, Alabama, ***U.S.A.*** (2000)

2. International Cancer Alliance - Symposium on Breast Cancer, Suburban Hospital, Bethesda, Maryland, ***U.S.A.*** (1999)

3. Department of Biology, University of York, York, England, ***U.K.*** (1999)

4. Inova Fairfax Cancer Center, Inova Fairfax Hospital, Falls Church, Virginia, ***U.S.A.*** (1999)

5. 9th Breast Cancer Think Tank, Sugar Bay, St. Thomas, ***U.S. Virgin Islands*** (1999)

B. Other Professional Activities

Study Section Memberships and Other Grant Reviews

1. Mail Reviewer, Northern Ireland Health and Personal Services Research and Development Strategy, Belfast, Northern Ireland, United Kingdom (2000).

2. Member, California Breast Cancer Research Program Study Section "Etiology & Prevention" (1999).

3. Member, U.S. Army Medical Research and Materiel Command Clinical and Experimental Therapeutics-1 study section.

4. Member, N.I.H. Grant Review Study Section "Chemical Pathology: Oncological Sciences Initial Review Group Special Emphasis Panel" ZRG2 SSS-1.

5. Member, American Institute for Cancer Research grant review study section "Panel I".

6. Member, Cancer Research Foundation of America's grant review panel.

Editorial Board Memberships (current)

1. Member, Editorial Advisory Board of the peer-review journal *Breast Cancer Research and Treatment.*

2. Member, Editorial Board of the peer-review journal *British Journal of Cancer*

3. Member, Editorial Board of the peer-review journal *Oncology Reports*

4. Assistant Editor of the peer-review journal *Journal of Mammary Gland Biology and Neoplasia*

C. Degrees

In 1999, the Principal Investigator was awarded the degree of Doctor of Science by his *alma mater*, The Queen's University of Belfast (United Kingdom). The DSc degree is awarded for a thesis containing significant research in breast cancer published since award of a PhD degree. In the U.K. and many other countries, a DSc is considered a "higher" degree than a PhD.

D. Funding Applied for Based on Work Supported by this Award

In direct support of this application, the PI successfully submitted an IDEA award to the DOD to fund studies into identifying the molecular mechanisms of antiestrogen resistance. In his role as mentor, the PI assisted and encouraged one of his predoctoral fellows to apply to the DOD for funding to study the role of EGF-receptor signaling in resistance to systemic therapies in breast cancer. This also was successfully funded. These applications are described below. As a collaborative venture with Dr. Steve Seeholzer (Principal Investigator) at Fox Chase Cancer Center, a further IDEA award was successfully submitted to the DOD to fund proteomic (not genomic) approaches to study antiestrogen resistance.

1. **IDEA Award BC990358:** "Molecular characterization of resistance." Dates: 01/06/00-01/06/03. Principal Investigator: Robert Clarke PhD. This award funds gene microarray analysis of antiestrogen responsive and resistant human breast cancer cell lines.

This application, which is one direct consequence of the additional time made available through this Academic Award, was successfully funded. Since the work is directly related to this Academic Award, which provides only salary support, the PI does not recover any salary support from the

IDEA award. The funded application does provide direct support for several of the studies described in the Academic Award.

2. **Predoctoral Fellowship BC990342:** "Modulation of epidermal growth factor receptor expression by chemotherapeutic agents in breast cancer cell lines". Dates: 01/06/00-01/06/03.
Principal Investigator (Predoctoral Fellow): James N. Welch; Mentor: Robert Clarke, PhD. This award funds studies into the role of altered EGF-R regulation as a potential survival response to chemotherapy. In addition, the Fellow has initiated studies into the possible role of EGF-R in resistance to antiestrogens, following preliminary data supported by this Academic Award demonstrating altered EGF-R mRNA expression in gene microarray studies of antiestrogen resistant breast cancer cells.

Conclusions

We have made considerable progress in addressing our proposed aims. The time made available to the PI through this Academic Award has resulted in several relevant publications and reviews, the ability to attract significant additional funding related to the research, and the generation of preliminary data that should lead to further publications in the coming year. The PI also continues to participate in other related professional activities. The time necessary to complete and successfully submit his DSc thesis, while not envisioned in the original application, also would not have been available had this award not been forthcoming. The PI will attempt to continue this level of productivity/activity in the remaining two years of support.

Appendices

Representative Reprints (reprints are not yet available for all publications)

J Natl Cancer Inst, 91: 46-53, 1999.

Clin Exptl Metastasis, 17:193-204, 1999.

Cancer Lett 136:134-151, 1999.

Br J Cancer 80:1682-1688, 1999.

Diseases of the Breast 2nd Edition, pp335-354, 2000.

Diseases of the Breast 2nd Edition, pp319-333, 2000.

Outline of submitted review

Reversal of Tamoxifen Resistance of Human Breast Carcinomas *In Vivo* by Neutralizing Antibodies to Transforming Growth Factor-β

Carlos L. Arteaga, Katri M. Koli, Teresa C. Dugger, Robert Clarke

Background: **Overexpression of transforming growth factor (TGF)-β has been reported in human breast carcinomas resistant to antiestrogen tamoxifen, but the role of TGF-β in this resistant phenotype is unclear. We investigated whether inhibition of TGF-β2, which is overexpressed in LCC2 tamoxifen-resistant human breast cancer cells, could modify antiestrogen resistance. *Methods:* TGF-β2 expression was evaluated in LCC2 cells and tamoxifen-sensitive LCC1 cells by northern blot analysis. Secreted TGF-β activity was quantified by use of an ^{125}I-TGF-β competitive radioreceptor assay. Sensitivity to tamoxifen was measured in a soft agarose colony-forming assay and in a xenograft model in nude and beige/nude mice. Natural killer (NK) cell cytotoxicity was measured by ^{51}Cr release from LCC1 and LCC2 cell targets coincubated with human peripheral blood mononuclear cells. Decrease in TGF-β2 expression in LCC2 cells was achieved by treatment with antisense oligodeoxynucleotides and confirmed by TGF-β2 immunoblot analysis. *Results and Conclusions:* The proliferative response of LCC2 cells to tamoxifen *in vitro* was not altered by TGF-β neutralizing antibodies. However, established LCC2 tumors in nude mice treated with tamoxifen plus TGF-β antibodies failed to grow, whereas tumors treated with tamoxifen plus a control antibody continued to proliferate. This reversal of tamoxifen resistance by TGF-β antibodies did not occur in beige/nude mice, which lack NK-cell function, suggesting that immune mechanisms may be involved in the antitumor effects of tamoxifen. Antisense TGF-β2 oligodeoxynucleotides enhanced the NK sensitivity of LCC2 cells in the presence of tamoxifen. Finally, LCC1 tumors were markedly more sensitive to tamoxifen in NK-active than in NK-deficient mice. *Implications:* These data suggest that host NK function mediates, in part, the antitumor effect of tamoxifen and that TGF-β2 may abrogate this mechanism, thus contributing to tamoxifen resistance. [J Natl Cancer Inst 1999;91:46–53]**

Transforming growth factors (TGF)-βs represent a large family of polypeptides involved in the regulation of cellular growth, differentiation, development, morphogenesis, and production of extracellular matrix *(1,2).* Three homologous mammalian isoforms have been reported: TGF-β1, TGF-β2, and TGF-β3. Although these isoforms overall share similar receptor-binding properties and biologic effects in multiple experimental systems *(1,2),* the mouse knockout phenotypes for each isoform are different *(3–5),* suggesting that their effects *in vivo* may not fully overlap. Expression of TGF-β1, -β2, and -β3 in human breast carcinoma cell lines and tumor tissues varies considerably [*see (6)*]. Several lines of data support the notion that mammary cell TGF-βs are autocrine regulators of tumor and nontumor breast epithelial cells *(7–9).* Although growth inhibition of breast tumor cells with antiestrogens is associated with enhanced secretion of TGF-β protein *(9),* transfection of a dominant negative type II TGF-β receptor into MCF-7 breast cancer cells does not abrogate the response to tamoxifen *(10),* suggesting that autocrine TGF-βs may not be universal mediators of tamoxifen action. On the other hand, several reports suggest that, in breast cancer cells, the TGF-βs are important mediators of tumor progression by fostering critical tumor/host cell interactions like the enhancement of peritumoral angiogenesis and stroma and the inhibition of mechanisms of immune surveillance [reviewed in *(6)*]. In some cases, overexpression of TGF-β1 has been temporally associated with estrogen independence and/or resistance to antiestrogens *(11–15).* Increased immunohistochemical staining for TGF-β1 protein in breast tumor sections is associated with shorter postmastectomy survival independent of other prognostic factors *(16).* We have studied the expression and function of TGF-βs in the tamoxifen-sensitive line LCC1 and the tamoxifen-resistant line LCC2 derived from MCF-7 human breast cancer cells. These cell lines were derived from MCF-7 mouse xenografts established in ovariectomized athymic mice. The LCC2 subline was selected *in vitro* in the presence of 4-hydroxytamoxifen. Both cell lines exhibit functional estrogen receptors (ERs) and progesterone receptors (PgRs) and form tumors in athymic nude mice in the presence or absence of added estradiol *(17,18).* This situation provides an experimental model to study mechanisms of tamoxifen *in vivo* resistance that do not involve loss of the ER.

Methods

Cell lines and reagents. The LCC1 (tamoxifen-sensitive) and LCC2 (tamoxifen-resistant) breast cancer lines were maintained in phenol red-containing improved minimal essential medium (IMEM; Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS; Hazleton Laboratories, Madison, WI) and 10 n*M* human insulin. Tamoxifen citrate was purchased from Sigma Chemical Co. (St. Louis, MO) and stored as a 1-m*M* stock solution in absolute ethanol. The 2G7 and 12H5 immunoglobulin G2 (IgG2) monoclonal TGF-β-specific antibodies (provided by B. M. Fendly; Genentech Inc., South San Francisco, CA) were generated against human recombinant TGF-β1 and have been described previously *(19).* The 12H5 IgG2 is devoid of TGF-β neutralizing activity, while the 2G7 antibody blocks growth inhibition by TGF-β1, -β2, and -β3 when tested against Mv1Lu mink lung epithelial cells *(19).*

Affiliations of authors: C. L. Arteaga, Department of Medicine, Vanderbilt University School of Medicine, Vanderbilt Cancer Center, Department of Veteran Affairs Medical Center, Nashville, TN; K. M. Koli, T. C. Dugger, Department of Cell Biology, Vanderbilt University School of Medicine, Nashville; R. Clarke, Lombardi Cancer Center, Georgetown University, Washington, DC.

Correspondence to: Carlos L. Arteaga, M.D., Division of Medical Oncology, Vanderbilt University School of Medicine, 22nd Ave., S., 1956 TVC, Nashville, TN 37232–5536 (e-mail carlos.arteaga@mcmail.vanderbilt.edu).

See "Notes" following "References."

RNA isolation and northern blot hybridization. Poly(A)$^+$ RNA was purified from subconfluent monolayer cultures using oligo-dT cellulose chromatography as described *(20)*. For northern blot analysis, 3 μg of messenger RNA (mRNA) was fractionated on 1.2% agarose gels containing formaldehyde and transferred to nylon membranes (Micron Separations Inc., Westboro, MA) by capillary transfer. Prehybridization and hybridization were performed at 42 °C in 50% formamide, 250 μg/mL single-stranded DNA, 1× Denhardt's solution, 50 μg/mL poly(A), 0.1% sodium dodecyl sulfate (SDS), and 5× standard saline citrate. Complementary DNA (cDNA) probes for TGF-β1 *(21)*, TGF-β2 *(22)*, and cyclophilin *(23)* were labeled with [^{32}P]deoxycytidine triphosphate (>3000 Ci/mmol; Amersham Life Science Inc., Arlington Heights, IL) using a Rediprime labeling kit (Amersham Life Science Inc.).

Preparation of cell-conditioned medium and TGF-β radioreceptor assay. Secreted TGF-β bioactivity was measured in serum-free IMEM conditioned for 24 hours by adherent breast cancer cells as described previously *(24)*. To activate secreted latent TGF-β, the conditioned medium was acidified with 1 N HCl to pH 1.5 for 1 hour at 4 °C and then neutralized with 1 N NaOH before testing in a TGF-β radioreceptor assay *(24)* utilizing AKR-2B (84A) mouse fibroblasts. Binding was performed in six-well plates in 1 mL/well binding buffer *(24)* containing 0.25 ng/mL. ^{125}I-TGF-β1 (specific activity, 173 μCi/μg; Du Pont NEN, Boston, MA) competed with variable volumes of conditioned medium. Human recombinant TGF-β1 (Genentech Inc.) was used to generate a standard curve from which the receptor binding activity of conditioned medium was calculated and then standardized to the number of cells from which the conditioned medium was collected. In this binding assay with AKR-2B (84A) cells, TGF-β1 and TGF-β2 are equipotent in displacing ^{125}I-TGF-β1 binding *(25)*. Addition of 1 μ*M* tamoxifen directly to the binding buffer had no effect on TGF-β1 binding in this assay (Arteaga CL: unpublished data).

Natural killer (NK) and lymphokine-activated killer (LAK) cell cytotoxicity assays. Sparse adherent cultures of LCC1 or LCC2 target cells (10^4 cells per well in a 96-well plate) were labeled for 4–6 hours at 37 °C with approximately 200 μCi/mL ^{51}Cr (specific activity, 400–1200 Ci/g; Du Pont NEN) in IMEM supplemented with 10% FCS. Unbound radioactivity was removed after two washes with growth medium before the addition of effector cells. Human peripheral blood lymphocytes (PBLs) from healthy volunteers were prepared by Ficoll–Hypaque gradient centrifugation. After two washes, variable numbers of NK effector cells were added to the ^{51}Cr-labeled breast cancer cell targets in a final volume of 0.2 mL/well in quadruplicate. To generate LAK effector cells, PBLs were incubated for 5 days at 37 °C in 5% CO_2 with 1000 U/mL human recombinant interleukin 2 (Chiron Therapeutics, Emeryville, CA) and then added in ratios ranging from 5 : 1 to 100 : 1 to freshly labeled tumor cell targets. Spontaneous ^{51}Cr release and PBL-induced (experimental) ^{51}Cr release were measured in both assays after an overnight coincubation, and percent cytotoxicity was calculated as described previously *(26)* by the formula: ([experimental release − spontaneous release]/total release) × 100.

Mouse studies. Five- to 8-week-old female athymic (nude) mice (Harlan Sprague-Dawley, Inc., Madison, WI) or NIH-III beige/nude mice (Taconic Farms, Inc., Germantown, NY) were inoculated subcutaneously just caudal to the forelimb with approximately 5 × 10^6 LCC1 or LCC2 tumor cells in 0.25 mL of serum-free IMEM via a 22-gauge needle. Variable times after tumor cell inoculation, 25-mg, 60-day release tamoxifen pellets (Innovative Research, Toledo, OH) were implanted subcutaneously in the back at a distant site from the tumor via a 10-gauge trocar. In some experiments, animals received daily intraperitoneal injections of 2G7 or 12H5 antibodies (100 μg each) in a 0.2-mL volume via a 26-gauge needle. Tumor diameters were measured serially with calipers, and tumor volume was calculated by the formula: volume = width^2 × length/2. At the completion of the experiments, some tumors were removed, fixed in 10% formalin, and paraffin-embedded, and 5-μm-thick sections were cut, stained with hematoxylin–eosin, and subjected to light microscopy. Care of all mice used in these studies was in accord with institutional guidelines.

Effects of antisense TGF-β2 oligonucleotides and immunoblotting of TGF-β2 secreted in cell-conditioned medium. TGF-β2 antisense phosphorothioate oligodeoxynucleotides as well as a nonsense oligonucleotide sequence of similar length and with the same GC content (used as control) were synthesized by and purchased from Ransom Hill Bioscience, Inc. (Ramona, CA), on the basis of a recent report *(27)*. A search in the European Molecular Biology Laboratory (EMBL) GenBank revealed no homologies between the 14-mer antisense sequence used in this study and the 5′ coding region of any known gene sequence. For inhibition of TGF-β2 expression, LCC2 cells were treated for 48 hours at 37 °C in serum-free IMEM with 3 μ*M* antisense or nonsense oligonucleotides. Cell medium was then collected, cleared of cell debris by centrifugation, concentrated approximately 30-fold in a Centriprep 30 MW column (Millipore Corp., Bedford, MA), boiled in Laemmli sample buffer containing the reducing agent dithiothreitol (DTT), and resolved by 15% SDS–polyacrylamide gel electrophoresis. Following transfer to nitrocellulose, membranes were subjected to an immunoblot procedure that utilizes a TGF-β2 polyclonal antiserum (R&D Systems Inc., Minneapolis, MN). Detection of TGF-β2 reactive bands in autoradiograms was performed with horseradish peroxidase-linked antirabbit immunoglobulins and enhanced chemiluminescence.

Statistical analyses. To analyze tumor growth curves, we used a repeated-measures analysis of variance (ANOVA) model with a serial correlation structure. The analysis was based on the logarithm of the tumor volume to stabilize the variance. To avoid strict assumptions about normality, the General Estimating Equation approach was used to fit the model, as implemented in SAS/PROC GENMOD software (SAS Institute, Inc., Cary, NC). The *in vitro* NK and LAK cytotoxicity data were analyzed using ANOVA, as implemented in SAS/PROC GLM software. For these cytotoxicity experiments, each data point represents the mean cpm of ^{51}Cr released from labeled target cells in four wells. If the standard deviations of the quadruplicate determinations were less than 10% of the mean value (before the experimental release was subjected to the formula for calculating % cytotoxicity [above]), they were not included in the figures.

Results

TGF-β Expression and Immune Sensitivity of LCC1 and LCC2 Breast Cancer Cells

We first examined steady-state mRNA levels of TGF-β1 and TGF-β2 in tamoxifen-sensitive and tamoxifen-resistant cells. LCC2 cells express similar low levels of TGF-β1 mRNA (not shown) but greater than 20-fold higher levels of TGF-β2 mRNA relative to LCC1 cells (Fig. 1, A). Protein levels in serum-free medium conditioned by LCC2 cells, as measured in a ^{125}I-TGF-β1 radioreceptor assay, were 6.8 ng of TGF-β equivalents/10^6 cells per 24 hours in the absence of acid activation *in vitro*, whereas LCC1 cells only secreted lower levels of latent TGF-β activity (Fig. 1, B). A 24-hour incubation with 1 μ*M* tamoxifen up-regulated TGF-β2 mRNA levels in LCC2 cells but not in LCC1 cells (Fig 1, A). By radioreceptor assay of serum-free cell medium, tamoxifen (1 μ*M*) treatment for 24 hours increased the secretion of (active) TGF-β activity to 13.9 ng/10^6 cells per 24 hours, whereas, in LCC1 cell medium, this bioactivity remained undetectable. To test the paracrine effects of TGF-β2 overexpression in the context of a multicellular experimental system, we measured NK and LAK sensitivity of ^{51}Cr-labeled LCC1 and LCC2 cells. NK and LAK cells are potently suppressed by exogenous TGF-β1 and TGF-β2 *(28)*. Consistent with these data and the overexpression of TGF-β2, LCC2 cells were statistically more resistant to NK cell-mediated lysis (P = .0001) and LAK cell-mediated lysis (P = .0002) than tamoxifen-sensitive LCC1 cells (Fig. 2).

TGF-β Neutralizing Antibodies and Sensitivity to Tamoxifen *In Vivo* and *In Vitro*

To study the role of TGF-β2 in tamoxifen resistance of LCC2 cells, we utilized the anti-TGF-β 2G7 IgG2 antibody, which neutralizes all three TGF-β mammalian isoforms *(19)*. Established subcutaneous LCC2 tumors in nude mice bearing 60-day-release, 25-mg subcutaneously implanted tamoxifen pellets were randomized to 100 μg/day of 2G7 or the 12H5 non-neutralizing anti-TGF-β control IgG2 by intraperitoneal injection. These systemic doses of 2G7 result in detectable and sustained plasma levels of an activity that blocks TGF-β receptor binding when tested *ex vivo (26)*. In animals treated with 2G7, but not with the

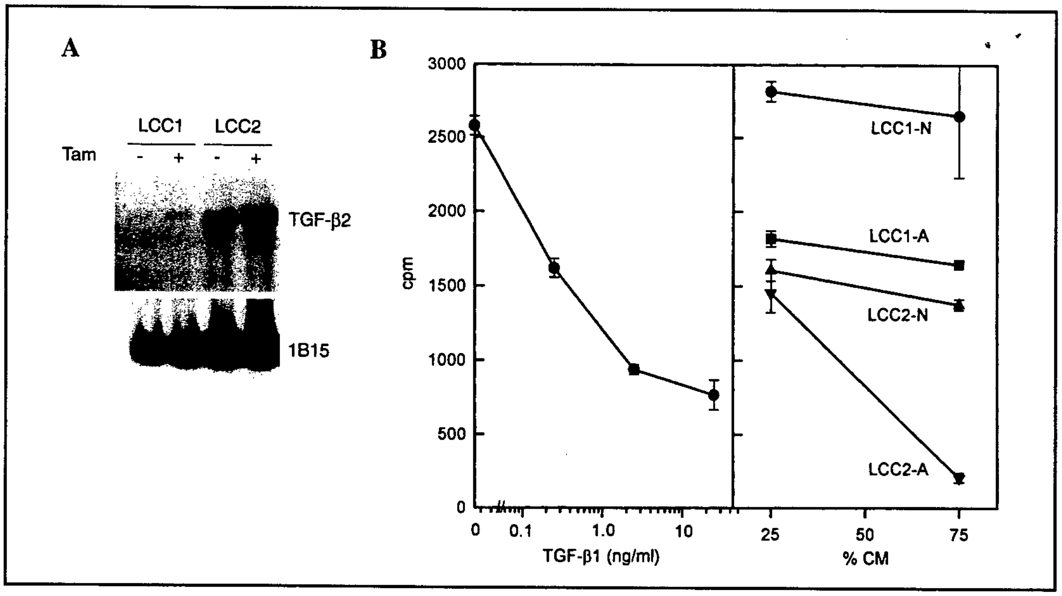

Fig. 1. A) Transforming growth factor (TGF)-β2 messenger RNA (mRNA) expression in LCC1 (tamoxifen-sensitive) and LCC2 (tamoxifen-resistant) human breast cancer cells. Poly(A)$^+$ mRNA was isolated by oligo-dT-cellulose chromatography. Where indicated, cells were treated with 1 μ*M* tamoxifen (Tam) for 24 hours prior to lysis and RNA collection. For northern blot analysis, 3 μg of mRNA per lane was fractionated on 1.2% agarose gels. After mRNA transfer, the nylon membranes were probed with [^{32}P]deoxycytidine triphosphate-labeled TGF-β2 and 1B15 (cyclophilin) probes as described in the "Methods" section. The 5.1-kilobase TGF-β2 transcript was overrepresented in LCC2 tumor cell RNA. **B)** Secretion of TGF-β activity into conditioned medium (CM). Serum-free conditioned medium was collected at 24 hours and tested in a 1-mL ^{125}I-TGF-β1 radioreceptor competition assay as described in the "Methods" section. **Left:** Standard curve using AKR-2B (84A) mouse fibroblasts and 0.25 ng/mL ^{125}I-TGF-β1 in the absence or presence of 0.25–25 ng/mL unlabeled TGF-β1. **Right:** Competing activity of 250 μL (25% conditioned medium of neutral [N] and 750 μL (75% conditioned medium) of transiently acidified (A) conditioned medium from LCC1 and LCC2 cells. When standardized on the basis of cell number, LCC2 cells secreted more than 10 ng/mL of TGF-β1 equivalents per 10^6 cells in a 24-hour period. Each data point represents the mean counts per minute (cpm) ± standard deviation of triplicate wells.

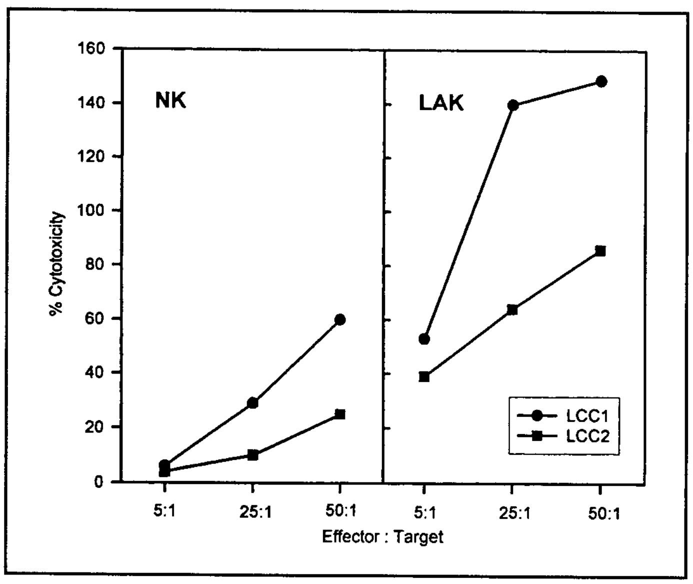

Fig. 2. Natural killer (NK) cell- and lymphokine-activated killer (LAK) cell-mediated cytotoxicity against LCC1 and LCC2 breast cancer cells. Breast cancer target cells were labeled with ^{51}Cr in improved minimum essential medium supplemented with 10% fetal calf serum in 96-well plates and then incubated overnight with human peripheral blood lymphocytes (PBLs) at the indicated effector-to-target ratios (for assessment of NK function). For assessment of LAK function, PBLs were incubated at 37 °C in 5% CO_2 with 1000 U/mL interleukin 2 (added only once) for 5 days and then coincubated with labeled target cells. Release of ^{51}Cr was measured to calculate percentage cytotoxicity as described previously *(23)*. Each data point represents the mean cytotoxicity calculated from four wells. NK, LCC1 versus LCC2: $P<.0001$; LAK, LCC1 versus LCC2: $P = .0002$ by analysis of variance.

12H5 control antibody, tumor growth was arrested (Fig. 3, A), suggesting that tumor cell TGF-β2 was mediating tamoxifen resistance in this experimental system. A second experiment yielded similar results. By light microscopy, both groups of LCC2 tumors were poorly differentiated adenocarcinomas with no major histologic differences between them. In the absence of tamoxifen treatment, 2G7 had no effect on LCC2 xenograft growth relative to 12H5-treated tumors, suggesting that tumor cell TGF-βs had no effect on basal LCC2 tumor growth (not shown).

Previous studies *(26–29)* have shown that systemic administration of anti-TGF-β antibodies can up-regulate NK function in nude mice that, in turn, exerts an antitumor effect. In addition, tamoxifen can induce and/or sensitize tumor cells to NK and LAK cells *in vitro* and *in vivo* (*discussed below*), thus implying that some of its antitumor effect is mediated by modulating this biologic response. These observations plus the high levels of NK activity present in athymic nude mice *(30)* and the relative resistance of LCC2 cells to NK activity (Fig. 2), a known cellular target for TGF-β2, suggested to us a model in which tamoxifen exerts some of its antitumor effect by up-regulating host NK function *in vivo*. Overexpression of immunosuppressive cytokines, like TGF-β2, can then counteract this tamoxifen-mediated host response and in part contribute to resistance to this antiestrogen. To test this hypothesis, we repeated the experiment shown in Fig. 3, A, in NK-deficient beige/nude mice. Neutralization of TGF-β2 with 2G7 did not restore tamoxifen sensitivity to LCC2 tumors in NK-deficient mice (Fig. 3, B), suggesting that, in this resistance model, NK host function is critical for the antitumor effect of tamoxifen *in vivo*.

To further support the hypothesis that tumor host mechanisms were involved in the restoration of tamoxifen sensitivity by neutralizing antibodies, we examined the effect of 2G7 in tamoxifen-treated LCC2 cells *in vitro*. In this cell-autonomous experimental system, LCC2 colony formation in the presence of 1 or 10 μg/mL 2G7 antibody was similar in the presence or absence of 1 μ*M* tamoxifen (Fig. 4). Tamoxifen stimulated LCC2 colony formation approximately 25% above ethanol (solvent for tamoxifen, used as control) in the presence of 1 or 10 μg/mL of the 12H5 control monoclonal antibody.

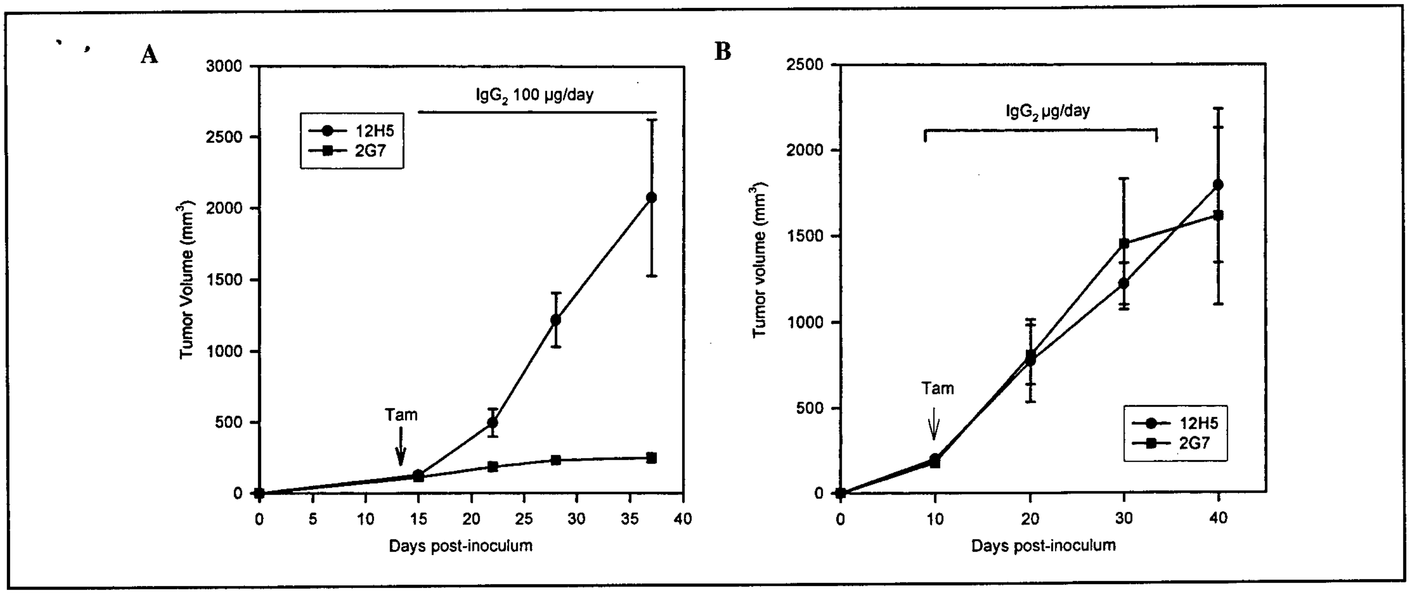

Fig. 3. Effect of anti-transforming growth factor (TGF)-β antibodies on tamoxifen (Tam) resistance of LCC2 breast cancer cell-induced tumors *in vivo.* **A)** Studies in athymic nude mice. Approximately 5×10^6 LCC2 cells were inoculated subcutaneously in the flank of female nude mice. On day 14, once the tumors had reached a volume of greater than 100 mm^3, all mice received a 25-mg, 60-day-release tamoxifen pellet subcutaneously at a site distant from the tumor. The following day, 100 μg/day of the 2G7 monoclonal antibody or the control immunoglobulin G2 (IgG_2) 12H5 was injected intraperitoneally, and antibody injections were continued daily for the next 3 weeks. Tumor diameters were measured serially with calipers and tumor volumes in mm^3 were calculated as described in the "Methods" section. Each data point represents the mean tumor volume ± standard deviation from six mice. 12H5-treated versus 2G7-treated tumor growth: $P<.0001$ by analysis of variance. **B)** Studies in NK-deficient beige/nude mice. NIH-3 beige/nude mice (NK-deficient) were inoculated subcutaneously with 5×10^6 LCC2 tumor cells. On day 10, once tumors had reached a volume of greater than 100 mm^3, tamoxifen pellets were implanted subcutaneously. The following day, daily intraperitoneal injections with 100 μg of the 2G7 or 12H5 monoclonal antibodies were started and continued for the next 3 weeks as in **panel A.** Each data point represents the mean tumor volume ± standard deviation from six mice.

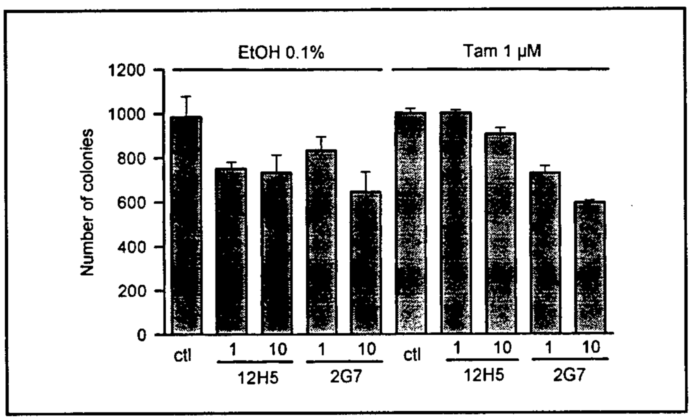

Fig. 4. Effect of neutralizing anti-transforming growth factor (TGF)-β antibodies on LCC2 breast cancer cell colony-forming ability *in vitro.* A single-cell suspension of 3×10^4 LCC2 tumor cells per dish were plated in soft agarose on 0.1% ethanol (EtOH) or 1 μ*M* tamoxifen (Tam; in 0.1% ethanol) as described in the "Methods" section. For each condition and where indicated, cells were coincubated or not (control [ctl]) with 1 or 10 μg/mL 12H5 or 2G7 antibodies. In the presence of either antibody, there were no differences in clonogenicity between tamoxifen-treated and ethanol-treated control cultures (P = .35; Pearson's x^2 test). Each data point represents the mean number of colonies ± standard deviation from three dishes.

Antisense Oligodeoxynucleotide-Mediated Inhibition of Tumor Cell TGF-β2 Expression and Enhancement of Tamoxifen-Induced NK Toxicity Against LCC2 Cells *In Vitro*

Several published data [*see (31)*] indicate that tamoxifen can stimulate immune effector cellular mechanisms in the tumor host as well as sensitize tumor cell targets to cytotoxicity independent of ER expression. Therefore, we first examined in a multicellular experimental system whether this sensitization to NK action was different in the tamoxifen-sensitive LCC1 versus tamoxifen-resistant LCC2 cells. Consistent with their phenotype *in vivo,* coincubation of NK effector cells with target cells with tamoxifen *in vitro* resulted in sensitization of the LCC1 cells (P = .0001). On the contrary, addition of tamoxifen reduced the lower level of NK-mediated ^{51}Cr release from LCC2 cells even further (P = .001; Fig. 5).

To test the contribution of TGF-β2 to NK resistance in the presence of tamoxifen, we used antisense phosphorothioate oligodeoxynucleotides *(27).* Treatment of LCC2 cells with 3 μ*M* antisense TGF-β2 oligodeoxynucleotides for 48 hours abrogated the secretion of TGF-β2, compared with nonsense oligodeoxynucleotides as measured by immunoblot of concentrated serum-free conditioned medium (Fig. 6). Proliferation of LCC2 cells was not affected during the 48-hour incubation with antisense oligonucleotides compared with nonsense oligonucleotide-treated cultures. We then examined the effect of antisense oligonucleotide-mediated inhibition of TGF-β2 expression in LCC2 cells on NK sensitivity in the absence and presence of tamoxifen. Treatment of LCC2 cells with TGF-β2 antisense oligonucleotides enhanced their sensitivity to NK effector cells in the presence of tamoxifen at all effector-to-target ratios tested, but not in the absence of tamoxifen (Fig. 7), suggesting that, in these tamoxifen-resistant cells, TGF-β2 mediates, in part, this relative resistance to NK activity and that down-regulation of TGF-β2 expression can unmask the NK-sensitizing effect of tamoxifen.

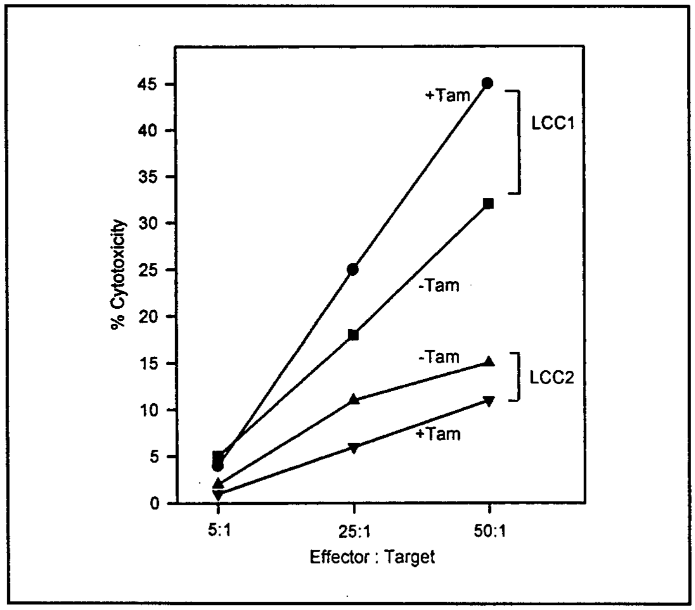

Fig. 5. Effect of tamoxifen (Tam) on natural killer (NK) cell activity against LCC1 and LCC2 breast cancer cells. Breast tumor cells (10^4 per well) in 96-well plates were labeled with ^{51}Cr in the presence or absence of 1 μM tamoxifen and then incubated overnight with peripheral blood lymphocytes at the indicated effector-to-target ratios in the presence or absence of an identical concentration of tamoxifen. Each data point represents the mean cytotoxicity derived from four identical wells. Tamoxifen-sensitive LCC1, but not tamoxifen-resistant LCC2 cells, were sensitized by tamoxifen to NK-mediated lysis. LCC1 with tamoxifen versus LCC1 without tamoxifen: $P<.0001$; LCC2 with tamoxifen versus LCC2 without tamoxifen: $P = .001$ (analysis of variance).

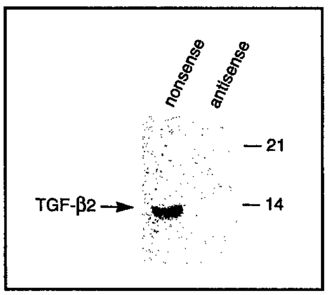

Fig. 6. Antisense-mediated inhibition of transforming growth factor (TGF)-β2 secretion. LCC2 breast cancer cells were treated for 48 hours in serum-free improved minimum essential medium in the presence of 3 μM nonsense or antisense TGF-β2 phosphorothioate oligodeoxynucleotides. Cell medium was collected, concentrated as described in the "Methods" section, and then subjected to an immunoblot procedure utilizing a TGF-β2 specific polyclonal antibody. Denaturation by sodium dodecyl sulfate and boiling in the presence of dithiothreitol result in reduction of the TGF-β2 active dimer to a monomeric TGF-β2 species of approximately 12.5 kD detectable in the medium from cells treated with nonsense oligodeoxynucleotides, but not antisense oligodeoxynucleotides.

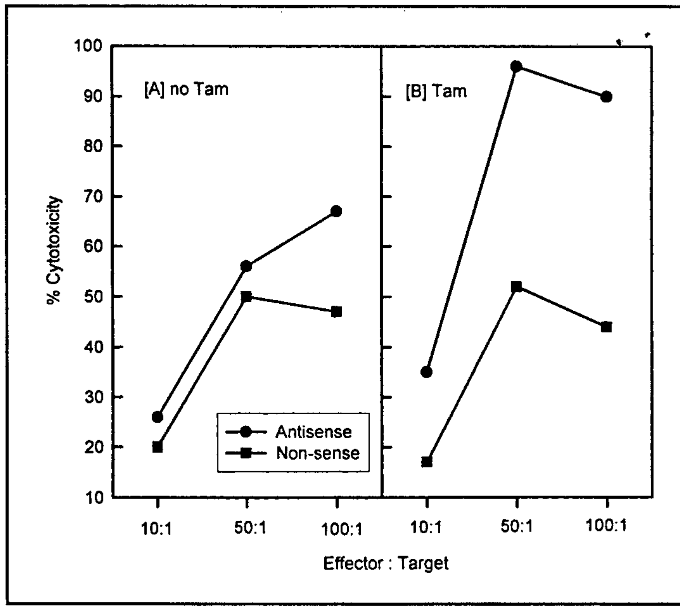

Fig. 7. Effect of transforming growth factor (TGF)-β2 antisense oligodeoxynucleotides on basal (absence of tamoxifen) and tamoxifen-stimulated natural killer (NK) cell function. LCC2 breast cancer cells were preincubated in serum-free improved minimum essential medium for 48 hours with 3 μM nonsense or antisense TGF-β2 oligodeoxynucleotides, and the cells were labeled with ^{51}Cr for the last 6 hours of this incubation period. After three washes, peripheral blood lymphocytes were added at the indicated ratios of effector-to-target cells, and isotope release from the target cells was measured after an overnight coincubation in the absence (**A**) or presence (**B**) of 1 μM tamoxifen (Tam) in RPMI-1640 medium supplemented with 10% fetal calf serum. Each data point represents the mean cytotoxicity calculated from four wells. All standard deviations from these quadruplicate determinations were less than 10%. Antisense oligodeoxynucleotide treatment of NK targets markedly enhanced LCC2 sensitivity to NK effector cells compared with nonsense oligonucleotide-treated cells when coincubated in the presence of tamoxifen ($P<.0001$ by analysis of variance).

Antitumor Effect of Tamoxifen in Nude Mice Versus Beige/Nude Mice Against Tamoxifen-Sensitive LCC1 Xenografts

To test whether both the growth-inhibitory effect and the sensitizing effect of tamoxifen on tumor targets to NK effector cells were related phenomena, we concurrently examined the inhibitory effect of tamoxifen on LCC1 tumors in nude mice, which exhibit elevated NK cell function *(30),* as well as in beige/nude mice. In these animals, the beige mutation markedly reduces NK function *(30).* Tamoxifen pellets were implanted 2 days after subcutaneous tumor cell inoculation. Within 4 weeks, 100% of LCC1 tumors formed in both nude and beige/nude mice without the need for estrogen supplementation and in the absence of tamoxifen treatment (Table 1). Four weeks after tumor inoculation, there were no detectable LCC1 xenografts in

Table 1. Antitumor effect of tamoxifen in nude versus beige/nude mice*

Host	Tamoxifen treatment	Animals with tumors/ total No. inoculated	Tumor volume, mm^3 ± SD†
Nude	—	8/8	123 ± 31
	+	0/8‡	N/A
Beige/nude	—	8/8	229 ± 65
	+	5/8§	83 ± 29

*10^7 LCC1 tumor cells/mouse were injected subcutaneously into 16 nude and 16 beige/nude mice as described in the "Methods" section. Two days later, eight mice per group received a subcutaneous, 25-mg, 60-day release tamoxifen pellet. Animals were serially followed for tumor formation and the tumors were harvested 4 weeks after inoculation. In the absence of tamoxifen treatment, tumor take was 100% in both groups. Five of eight beige/nude mice treated with tamoxifen developed subcutaneous xenografts, while none of the tamoxifen-treated nude mice exhibited measurable tumors at 4 weeks. Data shown represent the mean xenograft volume in mm^3 ± standard deviation of measurable tumors only. N/A = not applicable.

†SD = standard deviation.

‡Nude mice; tamoxifen-treated versus tamoxifen-untreated, two-sided $P<.001$.

§Beige/nude mice; tamoxifen-treated versus tamoxifen-untreated, two-sided $P = .20$ (Pearson's χ^2 test).

tamoxifen-treated nude mice. Conversely, five of eight NK-deficient beige/nude animals treated with tamoxifen exhibited subcutaneous tumors that measured more than 4 mm in diameter (>100 mm^3). The formation of LCC1 tumors suggested that, in this tumor model, host NK function was, in part, mediating the

growth-inhibitory effect of tamoxifen (Table 1). At 8 weeks, there were still no detectable LCC1 tumors in nude mice treated with tamoxifen.

Discussion

We have studied the role of TGF-βs in the tamoxifen-resistant phenotype of LCC2 human breast cancer cells. Hormone-dependent breast carcinoma cells follow a predictable pattern from antiestrogen sensitivity to the acquisition of antiestrogen resistance. A number of molecular and cellular mechanisms are involved in the emergence of this phenotype, including the (infrequent) loss of ER expression, the selection of ER mutants with altered transcriptional responses, alterations in the intracellular pharmacology and/or binding of tamoxifen to breast cancer cells, and other non-ER-related mechanisms (*see below*). Several reviews *(32,33)* cover discussions on these mechanisms in more detail. The experimental models employing LCC1 and LCC2 cells were used in this study. These cells maintain high ER levels and up-regulate PgR levels in response to exogenous estradiol *(17,18)*, thus providing a good system to study mechanisms of tamoxifen resistance that do not involve ER loss. Compared with other tamoxifen-resistant tumor models, LCC2 tumor growth is not stimulated by tamoxifen *(18)*.

LCC2 tamoxifen-resistant cells markedly overexpress TGF-β2 compared with LCC1 tamoxifen-sensitive cells. Tamoxifen resistance in LCC2 tumors was abrogated by the coadministration of the antiestrogen with neutralizing TGF-β antibodies when tested in nude mice. This response was not seen in beige/nude mice that lack NK activity. Furthermore, tamoxifen sensitized the tamoxifen-sensitive LCC1 cells, but not the resistant LCC2 cells, to NK effector cells *in vitro*. Antisense oligonucleotide-mediated inhibition of TGF-β2 secretion by LCC2 cells restored the ability of tamoxifen to sensitize LCC2 cells to NK cell-induced cytolysis. Finally, tamoxifen exhibited a greater antitumor effect against LCC1 tumor cells in NK-plus nude mice compared with that seen in NK-deficient beige/nude mice. Taken together, these data suggest that overexpression of TGF-β2 by breast tumor cells can mediate tamoxifen resistance *in vivo*. Our results also suggest that host NK function is involved in the antitumor effect of the antiestrogen tamoxifen and that overexpression of potently immunosuppressive cytokines, like TGF-β2, can counteract this antiestrogenic effect in the host and thus contribute to tamoxifen resistance. Of note, however, tamoxifen reduced the low level of NK cell-mediated toxicity against LCC2 cells (Fig. 5). This particular result may reflect the tamoxifen-mediated increase in TGF-β2 mRNA and the activation of secreted TGF-β activity in LCC2 cells (Fig. 1), which in turn can block NK cell function.

Our results would seem at odds with the dogma that proposes antiestrogen-induced up-regulation of autocrine TGF-βs in breast carcinoma cells and tumor tissues as a mediator of tamoxifen's antitumor action *(8,9)*, and that loss of this autocrine pathway can contribute to tamoxifen resistance. The LCC1/LCC2 model we used argues against this hypothesis in that tamoxifen-sensitive LCC1 cells bind TGF-β1 poorly and do not respond to exogenous TGF-β1. On the other hand, the LCC2 resistant cells express all three TGF-β receptors in high levels (data not shown) and exhibit responses to exogenous TGF-β1 in culture *(34)*. Furthermore, a more recent study by Koli et al. *(10)* also challenges this dogma, in that transfection of a suppressible dominant-negative truncated type II TGF-β receptor into anti-estrogen- and TGF-β-sensitive MCF-7 cells abrogated TGF-β responses but not tamoxifen-mediated growth inhibition.

A rise in either the plasma levels of TGF-β2 *(35)* or the tumor levels of TGF-β2 mRNA *(36)* has been observed in patients with metastatic breast tumors receiving tamoxifen therapy. In one of these studies, TGF-β1 and TGF-β3 expression in tumors was not altered by tamoxifen therapy *(36)*, suggesting a TGF-β isoform-specific effect of tamoxifen. It was proposed in these studies that up-regulation of TGF-β2 expression is a marker of clinical response to tamoxifen. However, the great majority of tamoxifen-sensitive mammary tumors will progress to a tamoxifen-resistant state, suggesting the possibility of a subsequent temporal association between breast tumor TGF-β2 overexpression and tamoxifen resistance. This is consistent with our findings in the LCC2 cells. TGF-β1 overexpression has also been temporally associated with antiestrogen resistance in human breast cancer cell lines *(11,12)* and mammary tumor tissues *(13,15)*.

The greater inhibition of tamoxifen-sensitive LCC1 tumors in nude mice, which have elevated NK and normal LAK activities, compared with that seen in beige/nude NK-deficient mice, suggests that the antiproliferative effect of tamoxifen and the sensitization to host NK (and possibly LAK) function are related phenomena. Natural cytotoxicity, mediated by NK cells, is believed to play an important role in host antitumor surveillance mechanisms. NK cells are predominantly large granular lymphocytes, the majority of which express CD16 and CD56 cell surface antigens and represent overall 5%–8% of PBLs *(37)*. NK cells can mediate cytolysis in the absence of major histocompatibility complex class 1 and class 2 antigen expression in target cells *(37,38)*. In patients with breast cancer and in experimental systems, tamoxifen has been shown to enhance NK function and/or increase the sensitivity of tumor cell targets in an ER-independent fashion *(31,39–42)*. However, in one report by Gottardis et al. *(43)*, prolonged treatment with tamoxifen inhibited NK function in nude mice and stimulated growth of tamoxifen-resistant MCF-7 tumors. Nonetheless, NK activity is inversely related to the clinical stage of disease and/or the presence of axillary lymph node metastases in patients with breast cancer *(44–46)*, implicating host natural cytotoxicity in the control of cancer progression. Accumulation of NK cells in tumors was temporally associated with a clinical spontaneous remission in a patient with metastatic breast carcinoma *(47)*. Other reports *(48–50)* indicate that pharmacologically achievable concentrations of tamoxifen enhance immune cytolysis of tumor targets mediated by LAK cells and cytotoxic T cells. Overall and except for the observation by Gottardis et al. *(43)*, these reports and our results are consistent with the hypothesis that host immune function may play a role in the antitumor effect of triphenylethylene antiestrogens, independent of the ER status of the tumor cell target. These reports also suggest the possibility of synergistic antitumor effects of tamoxifen with anti-TGF-β antibodies in other ER-negative neoplasms. This possibility remains to be tested.

In vitro, anti-TGF-β antibodies failed to reverse tamoxifen resistance in LCC2 cells. This result suggests that the observed reversal *in vivo* may not involve ER signaling in LCC2 cells. Other non-ER mechanisms of antiestrogen-mediated tumor inhibition that involve paracrine mechanisms have been reported. For example, tamoxifen and pure ER antagonists inhibit endothelial cell proliferation within mammary tumors and promote

the apoptosis of angiogenesis-dependent breast tumors *(51,52)*. Moreover, MCF-7 tumors transfected with fibroblast growth factor (FGF)-4 and FGF-1 develop hematogenous metastases *in vivo* and are unresponsive to tamoxifen *(53–55)*, suggesting a role for angiogenic factors in ER-independent resistance to tamoxifen. Of note, TGF-βs are potent inductors of angiogenesis *(2,56)*, thus leading to a possible causal association between overexpression of TGF-βs, high intratumoral microvessel density, and a tamoxifen-resistant phenotype. Whether enhanced TGF-β2-mediated angiogenesis is involved in the antiestrogen resistance of TGF-β2-overexpressing LCC2 tumors will require further experiments.

In summary, we have described a novel mechanism of antiestrogen resistance, as well as a potentially important immunologic component in tamoxifen response and acquired resistance in human breast carcinoma cells. It is interesting that the *in vitro* selection of LCC2 cells against tamoxifen produced a resistance mechanism that functions through an effect on cells or mechanisms (NK function) not involved in the initial selection. However, the greater effect of tamoxifen against LCC1 tumors in NK-competent versus NK-deficient mice, as well as the TGF-β2 antisense oligonucleotide-mediated sensitization of LCC2 cells to NK effectors *in vitro* in the presence of tamoxifen, strongly argues in favor of this immunologic component. In addition, the published effects of tamoxifen therapy on the expression or content of TGF-βs in patients' tumors and tumor models *(13,15,36)* suggest that tamoxifen-induced overexpression of TGF-βs can also occur *in vivo*. Several practical implications could be derived from these data. First, the effects of tamoxifen on tumor host (endothelial, immune) cells will be missed in cell-autonomous *in vitro* screens and potentially lead to false-negative preclinical models. Second, ER-positive tumors that overexpress TGF-βs may be clinically unresponsive *de novo* to tamoxifen and/or escape antiestrogens early and would, therefore, be candidates to alternative molecular therapies aimed at down-regulating the overexpressed immunosuppressive (or angiogenic) cytokines. Future prospective clinicoepidemiologic studies in patients should clarify the practical implications of these data and address how predictably the overexpression of TGF-βs by ER-positive breast tumors may determine clinical resistance to tamoxifen and other antiestrogens in general.

References

(1) Roberts AB, Sporn MB. The transforming growth factor-βs. In: Roberts AB, Sporn MB, editors. Handbook of experimental pharmacology. Heidelberg (Germany): Springer-Verlag; 1990. p. 419–72.

(2) Moses HL. The biological action of transforming growth factor β. In: Sara V, Hall K, Low H, editors. Growth factors from genes to clinical application. New York (NY): Raven Press; 1990. p. 141–55.

(3) Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, et al. Targeted disruption of the mouse transforming growth factor-β1 gene results in multifocal inflammatory disease. Nature 1992;359:693–9.

(4) Sanford LP, Ormsby I, Gittenberger-de Groot AC, Sariola H, Friedman F, Boivin GP, et al. TGFβ2 knockout mice have multiple developmental defects that are non-overlapping with other TGFβ knockout phenotypes. Development 1997:124:2659–70.

(5) Proetzel G. Pawlowski SA. Wiles MV. Yin MY. Boivin GP. Howles PN. et al. Transforming growth factor-β3 is required for secondary palate fusion. Nat Genet 1995;11:409–14.

(6) Koli KM, Arteaga CL. Complex role of tumor cell TGF-βs on breast carcinoma progression. J Mammary Gland Biol Neopl 1996;1:373–80.

(7) Pierce DF, Johnson MD, Matsui Y, Robinson SD, Gold LI, Purchio AF, et al. Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF-β1. Genes Dev 1993;7:2308–17.

(8) Knabbe C, Lippman ME, Wakefield LM, Flanders KC, Kasid A, Derynck R, et al. Evidence that transforming growth factor-β is a hormonally regulated negative growth factor in human breast cancer cells. Cell 1987;48: 417–28.

(9) Butta A, MacLennan K, Flanders KC, Sacks NP, Smith I, McKinna A, et al. Induction of transforming growth factor β_1 in human breast cancer *in vivo* following tamoxifen treatment. Cancer Res 1992;52:4261–4.

(10) Koli KM, Ramsey TT, Ko Y, Dugger TC, Brattain MG, Arteaga CL. Blockade of transforming growth factor-β signaling does not abrogate antiestrogen-induced growth inhibition of human breast carcinoma cells. J Biol Chem 1997;272:8296–302.

(11) Daly RJ, King RJB, Darbre PD. Interaction of growth factors during progression toward steroid independence in T-47-D human breast cancer cells. J Cell Biochem 1990;43:199–211.

(12) Hermann ME, Katzenellenbogen B. Alterations in transforming growth factor-β production and cell responsiveness during the progression of MCF-7 human breast cancer cells to estrogen-autonomous growth. Cancer Res 1990;54:5867–74.

(13) Thompson AM, Kerr DJ, Steel CM. Transforming growth factor β1 is implicated in the failure of tamoxifen therapy in human breast cancer. Br J Cancer 1991;63:609–14.

(14) Arteaga CL, Carty-Dugger T, Moses HL, Hurd SD, Pietenpol JA. Transforming growth factor β1 can induce estrogen-independent tumorigenicity of human breast cancer cells in athymic mice. Cell Growth Differ 1993;4: 193–201.

(15) Baillie R, Coombes RC, Smith J. Multiple forms of TGF-β_1 in breast tissues: a biologically active form of the small latent complex of TGF-β_1. Eur J Cancer 1996;32A:1566–73.

(16) Gorsch SM, Memoli VA, Stukel TA, Gold LI, Arrick BA. Immunohistochemical staining for transforming growth factor β_1 associates with disease progression in human breast cancer. Cancer Res 1992;52:6949–52.

(17) Brunner N, Boulay V, Fojo A, Freter CE, Lippman ME, Clarke R. Acquisition of hormone-independent growth in MCF-7 cells is accompanied by increased expression of estrogen-regulated genes but without detectable DNA amplifications. Cancer Res 1993;53:283–90.

(18) Brunner N, Frandsen TL, Holst-Hansen C, Bei M, Thompson EW, Wakeling AE, et al. MCF-7/LCC2: a 4-hydroxytamoxifen resistant human breast cancer variant that retains sensitivity to the steroidal antiestrogen ICI 182,780. Cancer Res 1993;192;3:3229–32.

(19) Lucas C, Bald LN, Fendly BM, Mora-Worms M, Figari IS, Patzer EJ, et al. The autocrine production of transforming growth factor β_1 during lymphocyte activation: A study with a monoclonal antibody-based ELISA. J Immunol 1990;145:1415–22.

(20) Sambrook J, Fritsch EF, Maniatis TE. Molecular cloning: a laboratory manual. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory; 1989.

(21) Derynck R, Jarrett JA, Chen EY, Eaton DH, Bell JR, Assoian RK, et al. Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells. Nature 1985;316:701–5.

(22) Madisen L, Webb NR, Rose TM, Marquardt H, Ikeda T, Twardzik D, et al. Transforming growth factor-β2: cDNA cloning and sequence analysis. DNA 1988;7:1–8.

(23) Danielson PE, Forss-Petter S, Brow MA, Calavetta L, Douglass J, Milner RJ, et al. p1B15: a cDNA clone of the rat mRNA encoding cyclophilin. DNA 1988;7:261–7.

(24) Arteaga CL, Coffey RJ Jr, Dugger TC, McCutchen CM, Moses HL, Lyons RM. Growth stimulation of human breast cancer cells with anti-transforming growth factor β antibodies: evidence for negative autocrine growth regulation by transforming growth factor β. Cell Growth Differ 1990;1:367–74.

(25) Lyons RM, Miller DA, Graycar JL, Moses HL, Derynck R. Differential binding of transforming growth factor-β1, -β2. and -β3 by fibroblasts and epithelial cells measured by affinity cross-linking of cell surface receptors. Mol Endocrinol 1991;2:1887–96.

(26) Arteaga CL, Hurd SD, Winnier AR, Johnson MD, Fendly BM, Forbes JT. Anti-transforming growth factor (TGF)-β antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity. Implications for a possible role of tumor cell/host TGF-β interactions in human breast cancer progression. J Clin Invest 1993;92:2569–76.

(27) Jachimczak P, Bogdahn U, Schneider J, Behl C, Meixensberger J, Apfel R, et al. The effect of transforming growth factor-β2-specific phosphorothioate-anti-sense oligodeoxynucleotides in reversing cellular immunosuppression in malignant glioma. J Neurosurg 1993;78:944–51.
(28) Ruscetti F, Varesio L, Ochoa A, Ortaldo J. Pleiotropic effects of transforming growth factor-β on cells of the immune system. Ann N Y Acad Sci 1993;685:488–500.
(29) Wallick SC, Figari IS, Morris RE, Levinson AD, Palladino MA. Immunoregulatory role of transforming growth factor β (TGF-β) in development of killer cells: comparison of active and latent TGF-β_1. J Exp Med 1990;172: 1777–84.
(30) Clarke R. Human breast cancer cell line xenografts as models of breast cancer. The immunobiologies of recipient mice and the characteristics of several tumorigenic cell lines. Breast Cancer Res Treat 1996;39:69–86.
(31) Baral E, Nagy E, Berczi I. The effect of tamoxifen on the immune response. In: Kellen JE, editor. Tamoxifen: beyond the antiestrogen. Boston (MA): Birkhauser; 1996. p. 137–78.
(32) Wiebe VJ, Osborne CK, Fuqua SA, DeGregorio MW. Tamoxifen resistance in breast cancer. Crit Rev Oncol Hematol 1993;14:173–88.
(33) Clarke R, Brunner N. Acquired estrogen independence and antiestrogen resistance in breast cancer. Estrogen receptor driven phenotypes? Trends Endocrinol Metab 1996;7:25–35.
(34) Arteaga CL, Dugger TC, Clarke R, Koli KM. Blockade of tumor cell transforming growth factor (TGF)-βs restores tamoxifen (tam) sensitivity to tam-resistant human breast cancer cells. Breast Cancer Res Treat 1996; 41:220A.
(35) Kopp A, Schmahl M, Knabbe C. Transforming growth factor β2 (TGF-β2) levels in plasma of patients with metastatic breast cancer treated with tamoxifen. Cancer Res 1995;55:4512–5.
(36) MacCallum J, Keen JC, Bartlett JMS, Thompson AM, Dixon JM, Miller WR. Changes in expression of transforming growth factor β mRNA isoforms in patients undergoing tamoxifen therapy. Br J Cancer 1996;74: 474–8.
(37) Trinchieri G. Biology of natural killer cells. Adv Immunol 1989;47: 187–376.
(38) Liao N, Bix M, Zijistra M, Jaenisch R, Raulet DH. MHC class I deficiency: susceptibility to natural killer (NK) cells and impaired NK activity. Science 1991;253:199–202.
(39) Screpanti I, Santoni A, Gulino A, Herberman RB, Frati L. Estrogen and antiestrogen modulation of the levels of mouse natural killer activity and large granular lymphocytes. Cell Immunol 1987;106:191–202.
(40) Mandeville R, Ghali SS, Chausseau JP. *In vitro* stimulation of human NK activity by an estrogen antagonist (tamoxifen) [letter]. Eur J Cancer Clin Oncol 1984;20:983–5.
(41) Berry J, Green BJ, Matheson DS. Modulation of natural killer cell activity by tamoxifen in stage I post-menopausal breast cancer. Eur J Cancer Clin Oncol 1987;23:517–20.
(42) Baral E, Nagy E, Berczi I. Modulation of natural killer cell-mediated cytotoxicity by tamoxifen and estradiol. Cancer 1995;75:591–9.
(43) Gottardis MM, Wagner RJ, Borden EC, Jordan VC. Differential ability of antiestrogens to stimulate breast cancer cell (MCF-7) growth *in vivo* and *in vitro*. Cancer Res 1989;49:4765–9.
(44) An T, Sood U, Pietruk T, Cummings G, Hashimoto K, Crissman JD. *In situ* quantitation of inflammatory mononuclear cells in ductal infiltrating breast carcinoma. Relation to prognostic parameters. Am J Pathol 1987;128: 52–60.
(45) Akimoto M, Ishii H, Nakajima Y, Iwasaki H. Assessment of host immune response in breast cancer patients. Cancer Detect Prev 1987;9:311–7.
(46) Horst HA, Horny HP. Characterization and frequency distribution of lymphoreticular infiltrates in axillary lymph node metastases of invasive ductal carcinoma of the breast. Cancer 1987;60:3001–7.
(47) Maiche AG, Jekunen A, Rissanen P, Virkkunen P, Halavaara J, Turunen JP. Sudden tumour regression with enhanced natural killer cell accumulation in a patient with stage IV breast cancer. Eur J Cancer 1994;30A: 1642–6.
(48) Baral E, Nagy E, Berczi I. Modulation of lymphokine-activated killer cell-mediated cytotoxicity by estradiol and tamoxifen. Int J Cancer 1996;66: 214–8.
(49) Baral E, Nagy E, Berczi I. Target cells are sensitized for cytotoxic T-lymphocyte-mediated destruction by estradiol and tamoxifen. Int J Cancer 1994;58:64–8.
(50) Albertini MR, Gibson DF, Robinson SP, Howard SP, Tans KJ, Lindstrom MJ, et al. Influence of estradiol and tamoxifen on susceptibility of human breast cancer cell lines to lysis by lymphokine-activated killer cells. J Immunother 1992;11:30–9.
(51) Gagliardi A, Collins DC. Inhibition of angiogenesis by antiestrogens. Cancer 1994;53:533–5.
(52) Haran EF, Maretzek AF, Goldberg I, Horowitz A, Degani H. Tamoxifen enhances cell death in implanted MCF7 breast cancer by inhibiting endothelium growth. Cancer Res 1994;54:5511–4.
(53) McLeskey SW, Kurebayashi J, Honig SF, Zwiebel J, Lippman ME, Dickson RB, et al. Fibroblast growth factor 4 transfection of MCF-7 cells produces cell lines that are tumorigenic and metastatic in ovariectomized or tamoxifen-treated athymic nude mice. Cancer Res 1993;53:2168–77.
(54) Zhang L, Kharbanda S, Chen D, Bullocks J, Miller DL, Ding IY, et al. MCF-7 breast carcinoma cells overexpressing FGF-1 form vascularized, metastatic tumors in ovariectomized or tamoxifen-treated nude mice. Oncogene 1997;15:2093–108.
(55) Zhang L, Kharbanda S, Hanfelt J, Kern FG. Both autocrine and paracrine effects of transfected acidic fibroblast growth factor are involved in the estrogen-independent and antiestrogen-resistant growth of MCF-7 breast cancer cells. Cancer Res 1998;58:352–61.
(56) Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, et al. Transforming growth factor type β: rapid induction of fibrosis and angiogenesis *in vivo* and stimulation of collagen formation *in vitro*. Proc Natl Acad Sci U S A 1986;83:4167–71.

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LCC15-MB: a vimentin-positive human breast cancer cell line from a femoral bone metastasis

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Abstract

The LCC15-MB cell line was established from a femoral bone metastasis that arose in a 29-year-old woman initially diagnosed with an infiltrating ductal mammary adenocarcinoma. The tumor had a relatively high (8%) S-phase fraction and 1/23 positive lymph nodes (LN). Both the primary tumor and LN metastasis were positive for estrogen receptor (ER) and progesterone receptor (PgR), but lacked *erb*B_2 expression. Approximately one year later, the patient presented with a 0.8 cm comedo-type intraductal mammary adenocarcinoma in the left breast that was negative for ER and PgR, but positive for *erb*B_2. Thirty-five months after the initial diagnosis she was treated for acute skeletal metastasis, and stabilized with a hip replacement. At this time, tumor cells were removed from surplus involved bone, inoculated into cell culture, and developed into the LCC15-MB cell line. The bone metastasis was a poorly differentiated adenocarcinoma lacking ER, PgR, and *erb*B_2, characteristics shared by the LCC15-MB cells, although ER can be re-expressed by treatment of the LCC15-MB cells for 5 days with 75 μM 5-aza-2′-deoxycytidine. The LCC15-MB cell line is tumorigenic when implanted subcutaneously in NCr *nu/nu* mice and produces long-bone metastases after intracardiac injection. Although the bone metastasis from which the LCC15-MB cell line was derived lacked vimentin (VIM) expression, the original primary tumor and lymph node metastasis were strongly VIM positive, as are LCC15-MB cells *in vitro* and in nude mice. The karyotype and isozyme profiles of LCC15-MB cells are consistent with its origin from a human female, with most chromosome counts in the hypertriploid range. Thirty-two marker chromosomes are present. These cells provide an *in vitro/in vivo* model in which to study the inter-relationships between ER, VIM, and bone metastasis in human breast cancer.

Introduction

Cancer of the breast is the most common form of cancer in women, and ultimately will affect 1 in 8 women in the United States [1]. It also is the second highest cause of cancer deaths overall, and in most cases, the mortality associated with breast cancer results from distant metastasis. Breast cancers metastasize to various visceral organs and brain, and this often causes acute and immediately life-threatening problems. However, approximately 85% percent of all breast cancer patients have detectable metastasis to bone at the time of autopsy [2–7]. Bome metastases can cause significant pain, spinal cord compression, and susceptibility to fracture. Furthermore, bone marrow aspirates indicate immunochemically detectable metastatic cells at the time of initial surgery in 27% of breast cancer patients [8], and this has recently emerged as a reliable indicator of metastatic disease and poor outcome [9]. Thus, bone metastasis represents an important aspect of breast cancer with substantial clinical significance.

Several lines of evidence indicate a molecular specificity to the process of bone metastasis, the most striking being the preference of the bone site for cells of breast and prostate tumor origin [7, 10]. However, mechanisms of bone metastasis are poorly understood, and it is clearly important to further elucidate the cellular processes necessary for human breast cancer cells to metastasize to, and destroy bone. Several *in vivo* models have been established in nude mice to elucidate factors involved in breast cancer metastasis. While both ectopic and orthotopic inoculation can result in distant metastatic spread, this rarely involves bone [11, 12]. Accordingly, seeding of mouse bones after intra-arterial injection has been developed as a model system for the study of bone metastasis, initially with melanoma cells [13], and more recently with breast cancer cells [14–16]. Using this model, Yoneda and co-workers have shown that MDA-MB-231 cells injected into the left ventricle reliably form osteolytic lesions in the bones of nude mice, and that these lesions can be stimulated by increased bone turnover and inhibited by bisphosphonates [16, 17]. Additional av-

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enues of research have examined the interactions between breast cancer cells and bone cells *in vitro* [18–20], and collectively these studies have provided some insights into the mechanisms at play in osteolytic bone metastasis [10, 21].

While studies in these models, and *in vitro*, rely on the existence and preservation of bone-metastasis-related traits in established breast cancer cell lines, none of the currently available breast cancer cell lines were derived from a bone metastasis. Most were derived from pleural effusions [22, 23], although BT-20 cells were derived from a primary mammary adenocarcinoma [24], Hs578T from a primary mammary carcinosarcoma [24], and the MDA-MB-361 from a brain metastasis [25]. We hypothesized that breast-to-bone metastasis derived cell lines may retain and recapitulate molecular and cellular aspects of this disease in animal models, and may subsequently provide a vehicle for their study. Thus, we now describe the establishment and initial characterization of LCC15-MB, a cell line isolated from the femoral metastasis of a breast carcinoma. Since LCC15-MB cells may have a better representation of attributes involved in bone metastasis, they may aid in the elucidation of the molecular mechanisms involved in this process. We have characterized LCC15-MB cells, the original primary tumor, and the bone metastasis from which this cell line was derived, for estrogen (ER) and progesterone (PgR) receptors and the intermediate filament protein vimentin (VIM), markers that have been correlated with bone metastasis and overall metastasis of breast cancer, respectively [26–28]. We also determined the ability of these cells to grow and recolonize bone in nude mice.

Materials and methods

Patient description and marker analysis

The description of each lesion and initial marker analysis at diagnosis are summarized in Table 1. The patient presented at age 28 with a 2.5 cm mass in the right breast. The mass was biopsied, diagnosed as infiltrating ductal carcinoma and removed, along with 23 lymph nodes, by modified radical mastectomy. The diagnosis was confirmed by mastectomy, and one of the 23 nodes found to contain metastasis. The biopsy, mastectomy, and positive node specimens were ER+, PgR+, and *erb*B_2-negative. An apparently unrelated primary lesion arose in the left breast approximately 11 months later, was biopsied, and subsequently removed by modified radical mastectomy. This contralateral tumor was ER-negative, PgR-negative, *erb*B_2+, and classified as a comedo-type ductal carcinoma *in situ*. This was thought to be the result of an independent event. Approximately 3 years after the initial primary tumor was diagnosed at another hospital, the patient presented at our institution with acute bone metastasis and was stabilized by hip replacement. Material from the bone metastasis was archived in paraffin, and also processed for propagation in culture. To our knowledge, the patient did not present with metastases to any additional organs.

Cell line isolation and culture

Surplus tumor material (approximately 1 cm^3) from inside the marrow cavity was collected in Richter's Improved Minimal Essential Medium (IMEM, Biofluids, Gaithersburg, MD), supplemented with antibiotics (penicillin and streptomycin; Biofluids, 100 U/ml final concentration), and transferred to the laboratory. The tissue was minced with scalpel blades into pieces of approximately 1 mm^3, and agitated vigorously in 50 ml IMEM with antibiotics to disperse loosely attached cells. The tissue pieces were allowed to settle, and the resultant supernatant plated in IMEM supplemented with 5% fetal bovine serum (IMEM/5%FBS). Each tumor piece was plated individually into 24-well clusters in IMEM/5%FBS, and cultures were incubated at 37 °C, 5% CO_2/ 95% air, in a humidified incubator. Media was replaced carefully every 3–4 days, and cultures were monitored microscopically. No viable cultures arose from plating the cell suspensions. The LCC15-MB culture arose from one of the 24-well explant cultures.

The LCC15-MB cells have since been subcultured and maintained under standard conditions routinely used for established human breast cancer cell lines, i.e., weekly passage at 1:10 in IMEM/10%FBS. The cells have been passaged continuously for approximately 50 passages, and were sent to the cell culture laboratory at the Children's Hospital of Michigan (Detroit, MI) for chromosomal analysis and characterization of isozyme phenotypes at passage 20.

Histology and immunocytochemistry

Archival paraffin blocks were obtained for both the biopsy and mastectomy of the original primary tumor in the right breast, the associated lymph node, the second (left side) tumor biopsy and corresponding mastectomy specimen, and the bone metastasis from which the cell line was derived. In addition, material from both subcutaneous xenografts and bone metastases arising in the nude mice were fixed in formalin and embedded in paraffin following routine procedure (after decalcifying bone specimens). Histological sections were prepared by the Lombardi Cancer Center Shared Tissue Resource. Slides were heated to 60 °C and submerged in xylene for 10 minutes (twice) to de-paraffinize tissue sections. After clearing in 100% and 70% ethanol, bones and tumor tissue were hematoxylin-eosin stained by routine procedures. Immunohistochemistry was performed on paraffin sections by autoimmunostainers (TechMate 1000, BioTek Solutions, Santa Barbara, CA and Ventana 320, Ventana Medical System, Tucson, AZ) using a standard peroxidase and avidin-biotin-complex method with diaminobenzidine as the chromogen. For immunohistochemistry, the estrogen receptor antibody (Immunotek, Inc. Westbrook, ME, USA) was used at a 1:50 dilution, the progesterone receptor antibody at 1:25 (Novacastra Laboratories, Ltd. Newcastle upon Tyne, UK), and the *erb*B_2 antibody at 1:75 (Immunotek).

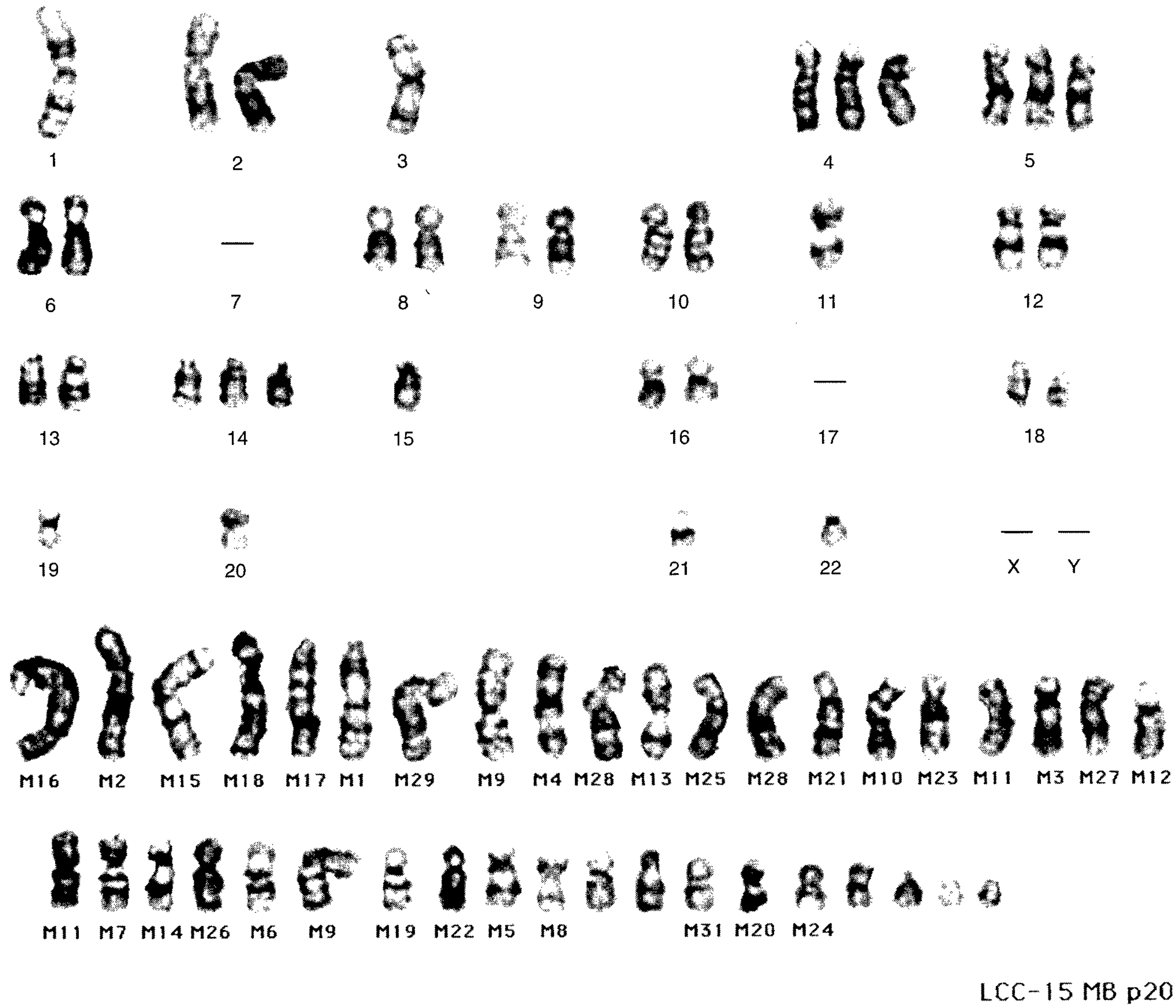

Figure 1. A representative karyotype prepared from the LCC15-MB cell line metaphases showing a total of 74 chromosomes. Five other karyotypes (not shown) revealed 73($n = 1$), 76($n = 1$) and 75($n = 3$) chromosomes respectively.

Immunofluorescence microscopy of cell cultures

Human breast cancer cells were plated on glass cover slips, placed in 24-well plates (Falcon/Becton Dickenson, Franklin Lakes, NJ), and cultured to approximately 80% confluence in IMEM/10% FBS. Individual cover slips were fixed in cold 80% methanol for 5 minutes, followed by cold acetone for 2 minutes. Before immunostaining, the cells were washed/rehydrated in 1X phosphate buffered saline (PBS, Gibco BRL, Gaithersburg, MD) and nonspecific sites blocked by incubation with 3% FBS for 30 minutes at room temperature. The coverslips were then incubated with primary antibodies against either vimentin (1:20 dilution, clone V9, Boehringer Mannheim, West Germany) or keratin (1:20 dilution, clone AE1/AE3, Gibco, BRL) diluted in 0.3% BSA for 1 hour at room temperature. A FITC-conjugated goat anti-mouse IgG secondary antibody (Gibco BRL, Gaithersburg, MD; dilution 1:50) was applied to the cells for 1 hour at room temperature. Coverslips were mounted on microscope slides and examined using a Zeiss Photoscope at a magnification of 25X.

Reverse transcription (RT) PCR analysis of keratins

The RT reaction was carried out using 500 ng RNA in each tube (total volume = 20 μl) with first strand buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$), 2.5 mM dNTPs, 10 mM DTT, 2.5 μM random hexamers, 1 U/ml RNAsin and 200 U MMLV reverse transcriptase (all reagents from Gibco BRL, Gaithersburg, MD). Cycle conditions were 1 hour at 42 °C and 5 minutes at 96 °C (Bios Thermal Cycler, Columbia, MD). The subsequent PCR amplification was carried out in a 50 μl reaction volume consisting of reaction buffer (50 mM KCl, 10 mM Tris-Cl, pH 8.8, 1 mM MgCl), 2.5 mM dNTP's, 0.5 μM primers, and 2.5 U Taq DNA polymerase (Gibco BRL, Gaithersburg, MD). The PCR cycles were 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C; 40 cycles total. The primers used are as follows: K18 (forward) 5′ GAA GAA CCA CGA AGA GGA AG 3′, K18 (reverse)

Table 1. Specimen description of the primary breast lesions and resultant lymph node and bone metastasis with estrogen receptor (ER) and progesterone receptor (PgR) status as determined by immunocytochemical assays at times of biopsy (Nichols Institute, San Juan Capistrano, CA). ND = no data.

Date	Tissue	Operation	Diagnosis	ER/PgR/Ploidy/S-Phase
1/11/90	Right breast	Biopsy	Infiltrating ductal carcinoma, 2.5 cm	ER = 30% cells positive PgR = negative Aneuploid (1.39) S-phase 8%
1/23/90	Right breast	Modified radical mastectomy	Residual intraductal carcinoma	ND
			1/23 lymph node metastases	ER 28 fmol/mg protein PR 121 fmol/mg protein Aneuploid (1.49) S-phase 11.2%
12/4/90	Left breast mass	Biopsy	Intraductal carcinoma (comedo-type)	ER = negative PgR = negative
12/18/90	Left breast	Modified radical mastectomy	Residual intraductal carcinoma No lymph node metastases	ER = negative PgR = negative
1/22/93	Bone, proximal femur	Biopsy	Metastatic adenocarcinoma	ER = negative PgR = negative

5′ CCA AGG CAT CAA GAT TA 3′ to amplify a 548 bp fragment of keratin-18 [29], and K19 (forward) 5′ AGG TGG ATT CCG CTC CGG GCA 3′, K19 rev 5′ ATC TTC CTG TCC CTC GAG CA 3′ to amplify a 460 bp segment of keratin-19 as described by Noguchi et al. [30]. Following PCR amplification, 10 μl of each PCR product was resolved on a 2% agarose gel and stained with ethidium bromide.

Estrogen receptor (ER) and progesterone receptor (PgR) analysis

ER and PgR data for the original primary breast tumor and lymph node metastasis were recovered from the original reports and confirmed in the archival paraffin blocks. We also performed immunohistochemistry on the LCC15-MB tumor xenograft and mouse bone metastasis. For the cell line *in vitro*, we performed ligand binding analysis for both ER and PgR [31]. We also performed RT-PCR analysis of ER before and after treatment with the demethylating agent 5-aza-2′deoxycytidine [32]. MCF-7 cells were included as a positive control. Cells were seeded at 5×10^3 cells/cm^2 in each of four T-175cm^2 flasks in IMEM/5%FBS. After 24 hours, the media from all flasks were replaced with IMEM/5%FBS without (control) and with (test) 0.75 μM 5-aza-2′deoxycytidine (Sigma, St. Louis, MO) for 5 days [32]. Total RNA was isolated with TRIzol reagent, and used as the template for cDNA synthesis by AMV reverse transcriptase. Human ER intron-spanning oligonucleotide primers (5′-GCACCCTGAAGTCTCTGGAA-3′ and 5′-TGGCTAAAGTGGTGCATGAT-3′) were used to amplify ER fragments by PCR. Cycling conditions for PCR, after an initial denaturation at 95 °C for 3 min, were 95 °C for 1 min, 55 °C for 30 seconds, and 72 °C for 1 minute for 40 cycles. Finally, one polymerization cycle at 72 °C for 5 minutes was performed. PCR products (20 μl of each 50 μl reaction) were size fractionated by electrophoresis in a 1% agarose gel.

In vivo *studies*

All experiments were performed in accordance with federal government and institutional guidelines for the humane use of vertebrate animals in biomedical research. Mice were housed in sterile laminar flow rooms at 25 °C and 50% humidity, and sacrificed by CO_2 asphyxiation at the end of each experiment. Subcutaneous (s.c.) xenografting was performed as previously described [33]. LCC15-MB cells were harvested from near confluence with trypsin, resuspended in phosphate buffered saline (PBS, Gibco BRL, Gaithersburg, MD) at 1×10^7 cells/ml, and inoculated bilaterally (5×10^6 cells/site) into the mammary fat pad region of 6-8-week-old ovariectomized female NCr *nu/nu* nude mice (NCI, Frederick, MD). Mice were monitored daily, and tumor measurements were taken twice weekly for 30 days. At sacrifice, samples of all organs were fixed in formalin, embedded in paraffin, and analyzed by routine histology (hematoxylin and eosin).

Intracardiac cell (i.c.) inoculation also was performed to examine the propensity of these cells for bone metastasis [15]. LCC15-MB tumor cells were harvested with trypsin as described above, washed twice by centrifugation, and resuspended (100,000 cells/100 μl) in PBS. Cell viability was determined by trypan blue exclusion and cell suspensions showed a viability greater than 90%. Prior to injection, the

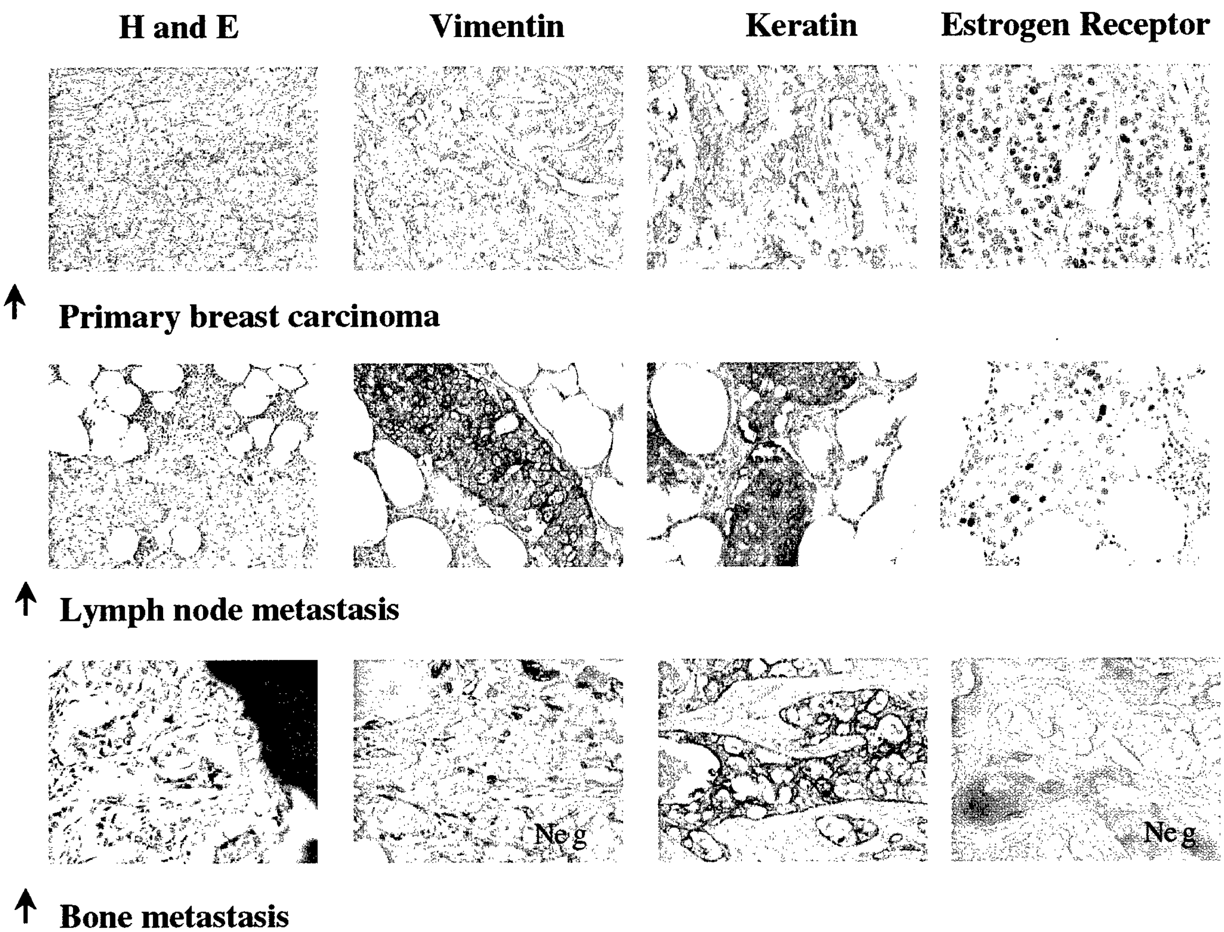

Figure 2. Immunocytochemical analysis of LCC15-MB (patient) right primary lesion, lymph node metastasis and bone metastasis. Routine hematoxylin and eosin (H & E), vimentin, keratin and estrogen receptor are shown. Note that tumor cells from right primary lesion and lymph node metastasis are ER-positive, while the bone metastases has lost ER expression of these markers. Vimentin was strongly positive in the original primary tumor and lymph node, but the focal staining in the bone metastasis was not reproducible and was considered negative. Keratin was strongly positive in all lesions.

cell suspension was agitated using a pipet to disperse aggregates. For i.c. injection of tumor cells into the left ventricle, animals were anesthetized by inhalation of methoxyflurane (Pittman Moore, Mundelein, NJ). The anterior chest was scrubbed with ethanol and the left ventricle located by using the second intercostal space and sternum as landmarks. A 30-gauge needle with a tuberculin syringe was inserted, with the spontaneous, pulsatile entrance of oxygenated blood into the needle hub indicating proper positioning of the needle. The tumor cells were then injected, and the mice allowed to recover from the anesthesia before being returned to their cages. After a period of four weeks, animals were sacrificed, bones and soft organs removed and placed in 10% formalin fixative for histological analysis. A total of nine mice per group was used in the experiment.

Results

Karyotype and isozyme analysis

Karyotype and isozyme analyses were performed in order to confirm the human origin and uniqueness of the LCC-15MB cell line. A representative chromosomal spread, from six such spreads, is shown in Figure 1. Table 2 shows the distribution of the normal and marker chromosomes, respectively. There were no normal sex chromosomes, although marker chromosome M25 appeared to be X-derived (Xq+). Chromosomes 7 and 17 were not present intact. There was one copy each of chromosomes 1,11,13,19, 21 and 22, one to two copies each of chromosomes 3, 10, 12, 15, 16 and 20, two copies of chromosomes 2, 6, 8, 9, and 18, two to three copies of chromosome 4, and three copies of chromosome 5 and 14. Only human chromosomes were detected, confirming that cells from other species were absent. The karyotype is consistent with that of a human female, with most chromosome counts in the hypertriploid range. The pattern appears unique, and is consistent with the cells being free of contamination with other cell lines. The isozyme profile of the LCC-15MB cells, as compared with other selected human

Table 2. Karyotype analysis of marker chromosomes for the LCC15-MB cell line. Thirty-two marker chromosomes were found and their probable origins and frequencies of occurrence as observed in the six karyotypes are summarized.

Marker and Probable Origins	Frequency per Karyotype (k)
M1 = inv.? (1qtr>1q22::1p35>1p10:)	6k
M2 = 2q+	6k, 2 copies / 3k
M3 = del (3) (p12;)	6k
M4 = iso (3q)	5k
M5 = ?Peri inv. (11) (p14q12)	6k
M6 = t(6p;?)	6k
M7 = inv. (7) (p14921)	6k
M8 = t (17q;?)	6k
M9 = t(7q;?hsr)	6k, 2 copies / 1k
M10 = (9q;10q)	6k
M11 = t (7p;10q)	6k, 2 copies / 4k
M12 = ?	6k
M13 = t (11q;15q?)	4k
M14 = del (11) (p11)	5k
M15 = t? (3pter>p24::hsr::p22>	6k
M16 = hsr?	6k
M17 = t (1pter>1p31::?)	6k
M18 = t? (3pter>3q21::4q22>4qtr)	5k
M19 = del (7) (:p14>q21::q32>qter)	6k
M20 = del(12) (q23)	5k
M21 = ?	5k
M22 = 13p+?	5k
M23 = 16q+?	6k, 2 copies / 4k
M24 = ?	6k
M25 = Xq+	6k
M26 = ?	3k
M27 = ?	6k
M28 = ?	4k, 2 copies / 2k
M29 = ?	3k
M30 = DEL (6) (P22.1)	4k
M31 = ?	4k
M32 = ?	5k

breast cancer cell lines, is shown in Table 3. Consistent with the karyotype analysis, it is apparent that the LCC-15MB cells represent a unique human cell line. The estimated frequency of this isozyme pattern is $P = 0.0432$, indicating that less than 5% of cell lines would be expected to have this profile. When considered with the karyotype analysis, it is apparent that the LCC-15MB cell line is unique and not contaminated with cells from other cell lines.

Hormone receptor status of LCC15-MB cells

Given the importance of hormone receptor status for breast cancer prognosis [26] and the presence of estrogen receptors in the original invasive mammary tumor and lymph node metastasis (Table 1), we examined ER and PgR levels in the bone metastasis from which LCC15-MB cells were derived. Retrospective immunocytochemical analysis of this patient material revealed it to be ER negative, PgR negative, and *erb*B_2 negative (Figure 2 and Table 4). While there is some evidence of focal vimentin staining in the bone metastasis

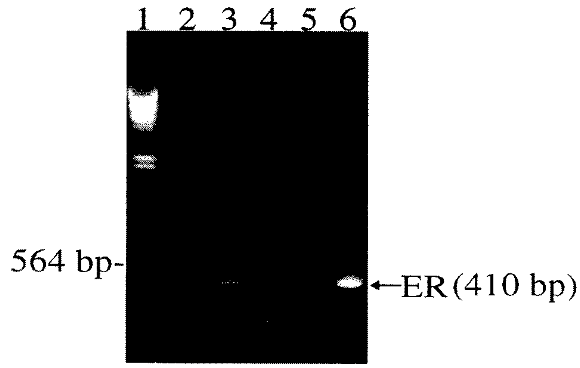

Figure 3. Ethidium bromide-stained agarose gel with bands representing estrogen receptor (ER) analysis of the LCC-15-MB cell line by RT-PCR, with reexpression of the ER in culture following addition of 5-aza-2′-deoxycytidine. Lane 1. Lambda/Hind III DNA markers (3 μg), 2. LCC15-MB (no treatment), 3. LCC15-MB (treated), 4. MDA-MB-231 (no treatment), 5. MDA-MB-231 (treated), 6. MCF-7.

(Figure 2), this is not reproducible and does not appear to represent authentic vimentin staining. The biopsy specimen from the right breast mass was positive for ER but negative for PgR (Table 1, Figure 2). In contrast, the lymph node metastasis was positive for both ER and PgR (Figure 2 and Table 4). ER and PgR were absent from the left breast biopsy and left mastectomy.

ER was not expressed by xenografted LCC15-MB cells growing either subcutaneously or in the bone marrow cavity of nude mice (Table 4, Figure 7), or in cultured LCC15-MB cells (Figure 3). The apparent focal ER staining (Figure 7) is not reproducible and, since ligand binding (not shown) and RT-PCR analyses were negative (Figure 3), is most unlikely to reflect ER expression. ER mRNA expression could be obtained in the LCC15-MB cells by 5-aza-2′-deoxycytidine treatment for 5 days [32]. Figure 3 depicts RT-PCR results showing amplified cDNA fragments of the expected size (410 bp) in cDNA pools derived from LCC15-MB and MDA-MB-231 cells treated with 5-aza-2′ deoxycytidine (Figure 3, lanes 3 and 5), but not in the untreated cells.

In vitro *morphology and intermediate filament proteins*

We compared the morphological characteristics of LCC15-MB cells to cell lines in which the progression status has been well established [34–36]. As seen in Figure 4, the LCC15-MB cells more closely resemble the stellate morphology of the invasive MDA-MB-231 human breast cancer cell line, than the more polygonal, epithelial clusters formed by the MCF-7 cells. Such a stellate morphology in culture has been associated with the expression of vimentin [33, 34], an intermediate filament protein usually seen in mesenchymal cells. Vimentin expression is up-regulated in all invasive human breast cell lines we have studied to date [33, 36, 37]. Because of their stellate morphology, we analyzed expression of vimentin, and the epithelial intermediate filament protein counterpart, keratin, in LCC15-MB cells compared to several other human breast cancer cell lines. As seen in Figure 5, both the LCC15-MB and MDA-MB-435 cells strongly express vimentin, whereas keratin expression

Table 3. Isozyme analysis of several breast cancer cell lines including the MCF-7, MCF-7/MIII, MCF-7/ADR, MDA-MB-231, MDA435/LCC6, EVSA-T, LCC15-MB and ZR-75-1 human breast cancer cell (Human breast cancer) lines; LDH = lactate dehydrogenase, G6PD = glucose-6-phosphate dehydrogenase, PGM1 = phosphoglucomutase-1, PGM3 = phosphoglucomutase-3, ESD=esterase D, Me-2 = mitochondrial malic enzyme, AK-1 = adenylate kinase, GLO-1 = glyoxalase. From this analysis, the phenotypic frequency of the LCC15-MB cell line is 0.0432 signifying that less than 5% of other cell lines share an identical isozyme phenotype profile. ND = no data.

Cell Line	LDH	G6PD	PGM1	PGM3	ESD	Me-2	AK-1	GLO-1
MCF-7	Human-5	B	1	1	1	0	1	1–2
MCF7/MIII	Human-5	B	1	1	1	0	1	1–2
MCF7/ADR	Human 5	B	1	1	1	0	1	1–2
MDA-MB-231	Human-5	B	1–2	1	1	ND	1	2
MDA435/LCC6	Human-5	B	2	1	1	0	1	2
EVSA-T	Human 5	B	1	1	1	1	1	2
LCC15-MB	Human-5	B	1–2	1	1	0	1	2
ZR-75-1	Human-5	B	1–2	2	1	0	1	2

Table 4. Summary of immunocytochemical data for estrogen receptor (ER), and progesterone receptor (PgR), keratin (Ker), vimentin (Vim) and *erb*B_2 in the LCC15-MB primary breast lesions, resultant lymph node and bone metastasis, and subcutaneous and bone xenografts of LCC15-MB cells in nude mice. ER, PgR. Ker Vim and *erb*B_2 analyses in this table were done at Georgetown University, those in Table 1 were obtained commercially. ND = no data.

Tissue	Operation	Diagnosis	ER	PgR	Ker	Vim	*erb*B_2
Right breast mass	Biopsy	Infiltrating ductal carcinoma, 2.5 cm	+ (80%)	–	+	+	–
Right breast mass	Modified radical mastectomy	Residual intraductal carcinoma	+ (50%)	+ (30%)	+	+	–
		1/23 lymph node metastases	+ (90%)	+ (20%)	+	+	–
Left breast mass	Biopsy	Intraductal carcinoma (comedo-type)	–	–	+	–	+
Left breast mass	Modified radical mastectomy	Residual intraductal carcinoma, No lymph node metastasis	–	–	+	–	+
Bone, proximal femur	Biopsy	Metastatic adenocarcinoma	–	–	+	–	–
Subcutaneous xenograft			–	–	±	++	–
Bone metastasis, intracardiac			–	–	±	++	ND

is undetectable. Vimentin also is highly expressed in both the LCC15-MB subcutaneous tumor and bone metastasis (Table 4). In contrast, the relatively epithelioid MCF-7 cells show strong keratin but not vimentin staining. The MDA-MB-231 cell line expresses intermediate levels of each class of intermediate filament protein.

We were surprised at the lack of keratin immunostaining in the carcinoma-derived LCC15-MB and MDA-MB-435 cells, and further investigated keratin mRNA expression levels in these cell lines by RT-PCR (Figure 6). Specific primers to keratin 18 amplified the appropriately-sized fragment in both LCC15-MB and MDA-MB-435 cell lines, but this was not the case for keratin 19. In contrast, the MCF-7 and MDA-MB-231 cell lines expressed both keratin 18 and 19. Keratins 18 and 19 represent two of the more reliable markers for epithelial differentiation [38]. While keratin 19 expression is more restricted, both keratin 18 and 19 have been demonstrated in malignant and benign tissues [38, 39].

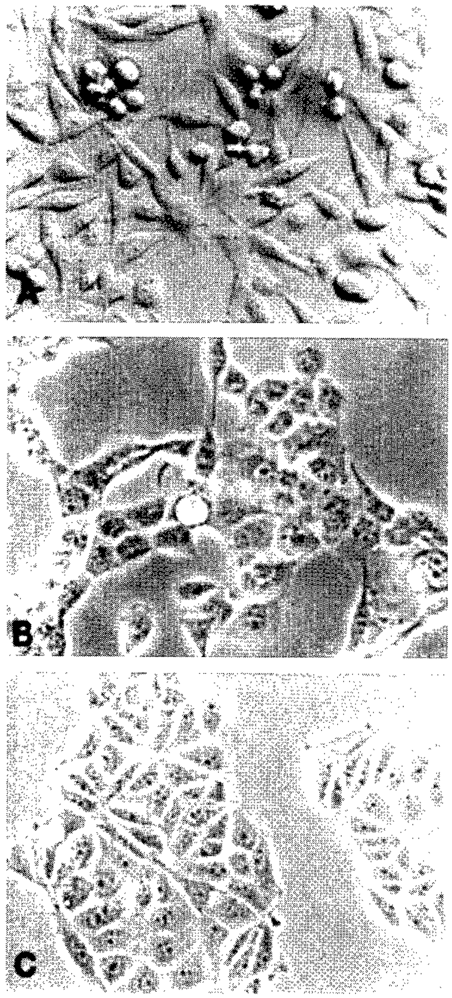

Figure 4. Phase contrast micrographs displaying typical morphology of the LCC15-MB (A), MDA-MB-231 (B) and MCF-7 (C) cell lines grown on plastic. Compare the stellate morphology of the MDA-MB-231 and LCC15-MB cells to the compact morphology of MCF-7 cells. Magnification = 40×.

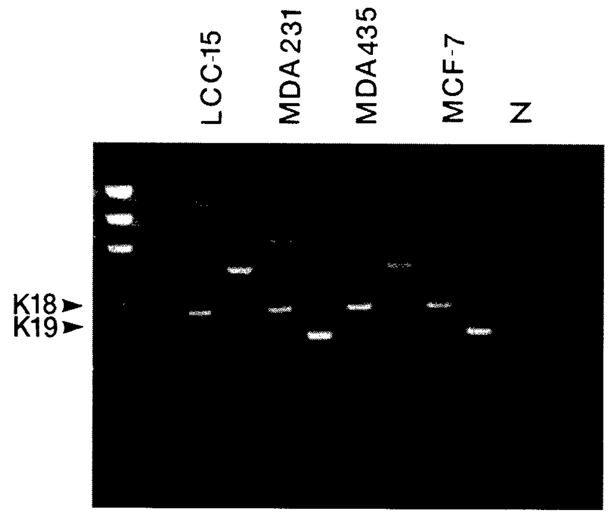

Figure 6. Ethidium bromide-stained agarose gel with bands representing expression of keratin 18 or 19 in LCC15-MB, MDA-MB-231, MDA-MB-435 and MCF-7 cells as determined by RT-PCR. Lane N is a negative control for the RT reaction in which the reverse transcriptase enzyme was omitted. Note that while keratin 18 is expressed by all four cell lines, keratin 19 is not expressed by the LCC15-MB or MDA-MB-435 cell lines.

The presence of keratin 18 mRNA in the LCC15-MB and MDA-MB-435 cell lines confirm their epithelial derivation, but the low overall levels of keratin protein (Figure 5), and the lack of keratin 19 mRNA, suggest that they have selectively lost epithelial characteristics while gaining a more mesenchymal phenotype.

We also evaluated *erb*B_2 levels. The right breast biopsy and modified radical mastectomy tissues were *erb*B_2 negative, as was the patient's bone metastasis and the LCC15-MB cells growing *in vitro* and *in vivo* (Table 4). In contrast, *erb*B_2 was detected in the intraductal carcinoma that arose in the left breast.

In vivo *growth*

Upon subcutaneous inoculation into nude mice, the LCC15-MB cells showed exponential growth after a lag phase of 7 days. The tumors have a doubling time of approximately 6 ± 2 days, with tumors arising in 100% of mice inoculated. The tumors are homogeneous, poorly differentiated carcinomas (Figure 7). Since LCC15-MB cells were derived from a bone metastasis, we also used a recently described model for bone metastasis analysis in nude mice [15–17]. LCC15-MB cells were injected i.c. in two separate experiments. In the first experiment, target organs were minced and cultured, and the metastasis to different organs scored on the basis of LCC15-MB cell outgrowth. As summarized in Table 5, LCC15-MB cells formed occasional lesions in a number of organs (kidney, spleen, lung, liver, heart and brain), but consistently colonized bone, as assessed by the outgrowth from cultured organs or bones resected from each mouse. In both the patient biopsy and the LCC15-MB xenografts, the bone lesions were predominately osteolytic. However, as might be expected, there is some evidence of new bone formation in the patient bone lesion.

One mouse developed an intraperitoneal tumor that may have been seeded through injection error. Outgrowth culture is the most sensitive indicator of viable metastasis in comparative studies using the MDA-MB-231 cells, which metastasize much less frequently to bone [15]. In the second experiment, we processed bone, spleen, kidney, brain, lung and liver for histological analysis four weeks after intracardiac injection. Histologically evident bone metastasis was apparent in one of the four bones sectioned, as illustrated in Figure 7. These data confirm the capability and propensity of this cell line for bone metastasis in this model.

Discussion

We have developed a new cellular model for the study of metastasis from human breast cancer in general, but particularly to bone. These cells are shown to be karyotypically distinct from other human breast cancer cell lines, but have a

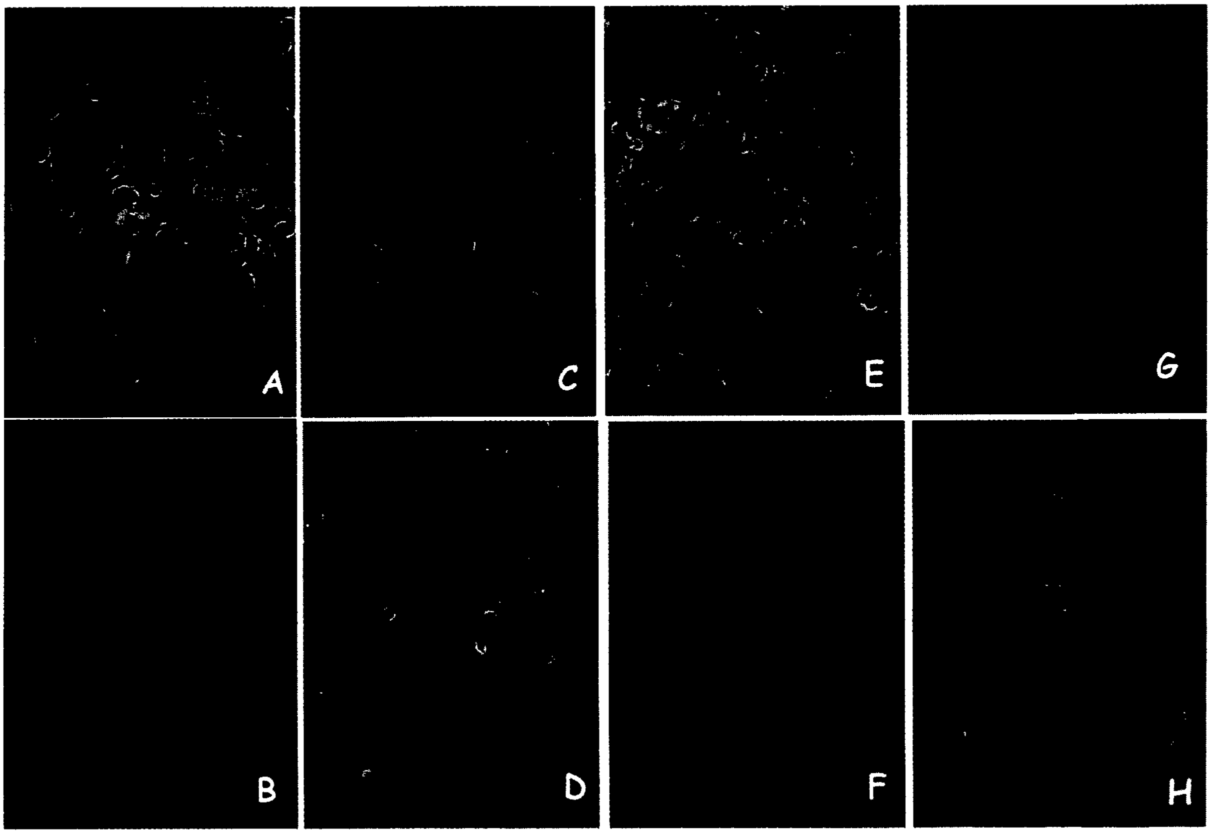

Figure 5. Micrographs showing immunofluorescent labeling of vimentin (A,C,E,G) and keratin (B,D,F,H) in LCC15-MB (A,B), MDA-MB-231 (C,D), MDA-MB-435 (E,F) and MCF-7 (G,H) human breast cancer cell lines. Although LCC15-MB and MDA-MB-435 cells stain rather strongly for vimentin, they display very little keratin. In contrast, MCF-7 cells express high levels of keratin, but no vimentin. The MDA-MB-231 cell line appears to express intermediate levels of both vimentin and keratin. Magnification = 25×.

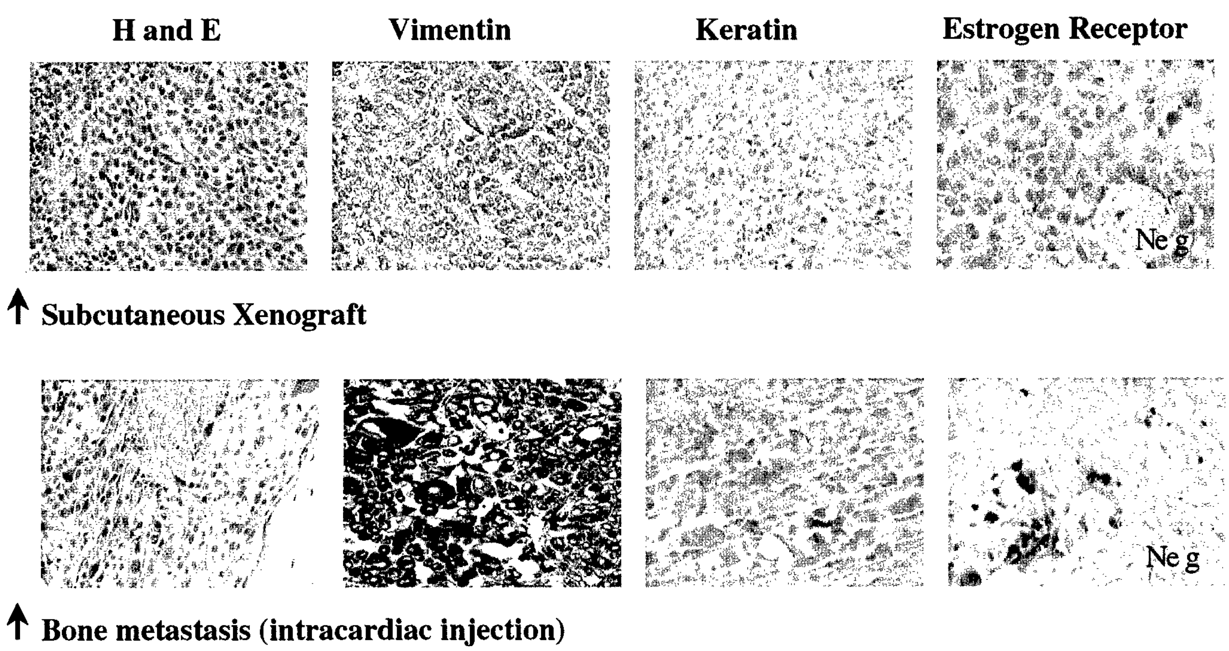

Figure 7. Immunohistochemical analysis of vimentin, keratin and estrogen receptor expression in the LCC15-MB cell line growing as a subcutaneous xenograft or bone metastasis resulting from inoculation of the cell line into nude mice. As seen in culture, the LCC15-MB cells remain positive for vimentin but negative for keratin and estrogen receptor at both sites. Background cytoplasmic staining for estrogen receptor is considered negative in the absence of nuclear staining, as indicated. Magnification = 10×.

Table 5. Frequency of organ specific metastasis from a representative LCC-15-MB cell intracardiac injection experiment (group of 5 mice) as measured by resultant cell cultures (total number established) obtained following sacrifice of the animal. Note that the majority of cultures, representing metastases (70%), arose in bony sites (tibia, femur, vertebra).

Organ site	Number of cultures
Tibia	4
Femur	4
Vertebra	8
Lung	0
Liver	0
Kidney	1
Spleen	2
Heart	1
Brain	1
Intraperitoneal tumor	1

mesenchymal-like phenotype indicated by vimentin expression, a hallmark shared by other metastatic cell lines. It is particularly valuable to have access to the patient material from which this cell line was established, and a number of relationships have been identified with respect to marker analysis in the primary tumor. The expression of ER in the primary tumor is consistent with the higher propensity reported for ER-positive tumors to metastasize to bone [40–42]. However, it was surprising to find that both the cell line and bone metastasis from which it was derived lacked ER expression, since the original primary tumor and lymph node metastasis were strongly positive. Further analyses on primary tumor and corresponding bone metastasis is required to clarify whether this case is anomalous, or whether ER is down-regulated in the bone environment after facilitating the initial metastasis.

Down-regulation of the ER in bone metastases has not been widely reported, though it has been hypothesized that the enhanced propensity of ER-positive breast cancer cells to metastasize to bone may be due to local steroid production, since bone cells are also estrogen-responsive [43–46]. It is not clear whether cells in the bone environment synthesize large amounts of estrogens. However, estrogen down-regulates ER expression, with prolonged exposure to any bone-derived estrogen possibly resulting in the hypermethylation of the ER gene as implicated by the 5-aza-2′ deoxycytidine data.

ErbB_2 is a member of the EGF-receptor family, and interacts with *erb*B_3 or *erb*B_4 to facilitate responses to heregulins [47]. In the current study, *erb*B_2 overexpression as detected by immunohistochemistry, was absent from all the specimens in the right breast. While this expression was clearly present in the intraductal carcinoma from the left side, it was not seen in either the bone metastasis, cell line, or xenografts. Amplification and/or overexpression of *erb*B_2 has been shown to correlate with poor clinical outcome in lymph-node positive patients, but the prognostic significance of *erb*B_2 in node-negative disease, as seen here in the left breast lesion, is controversial [48]. *Erb*B_2 expression has been associated with poor clinical outcome in a certain subset of node-negative patients; those with small, ER-positive, predominantly invasive tumors [49]. The lack of expression of *erb*B_2 in the metastasic bone lesion supports the underlying assumption that it arose from the *erb*B_2 negative, invasive lesion on the right, rather than from the DCIS on the left. Preliminary attempts to verify this assumption using FISH analysis with six commonly-altered markers were inconclusive.

The original primary tumor and lymph node metastasis were strongly VIM+, yet the bone metastasis in the patient was clearly VIM-negative. The possibility that VIM expression could be down-regulated by the bone environment was explored in the nude mouse experimental bone metastasis model, but VIM expression remained high. Analysis of a series of bone metastases in conjunction with their primary tumors is required to further explore this issue. The abundant expression of VIM in the primary tumor and lymph node metastasis is consistent with derivation from an aggressively metastatic tumor, since a strong correlation has been reported between basement membrane invasiveness *in vitro* and the expression of vimentin in cell lines [33]. Clinically, vimentin associates with a high growth fraction, poor nuclear grade, and lack of ER in breast cancer [50, 51], and so it is surprising to see coexpression of VIM and ER in the primary lesion and lymph node metastasis. We have previously seen that VIM is down-regulated in the lymph node metastases associated with VIM+ cervical carcinomas [52], but this has not occurred in the LCC15-MB patient, since the lymph node metastasis is strongly VIM+.

Vimentin expression has been reported in a variety of epithelial tumors, where it is thought to arise by a process resembling the epithelial to mesenchymal transition (EMT, reviewed in [28]). The EMT is known to transiently occur when epithelial cells adopt a migratory and possibly invasive state, during embryogenesis, organ development, and wound healing (reviewed in [53]). Domagala et al. [51] found that 22% of 253 invasive, ductal but not-otherwise-specified breast carcinomas were VIM positive. While there was no association between vimentin and lymph node status in this study, vimentin was an independent predictor of outcome in the node-negative group. In our own study of 60 invasive ductal breast carcinomas, we found focal vimentin expression in 6.7% of non-recurring and 26.7% of recurring tumors [54]. The EMT could contribute to the invasive progression of many more carcinomas, where it may be expressed by fewer cells in the tumor periphery [28]. Such expression has been difficult to verify because of the abundance of vimentin-positive host cells in this region. No previous data exist regarding the potential relationships between vimentin expression and bone metastasis, but our studies warrant further investigation in this area. Nevertheless, the data from this patient that expression of vimentin in the primary tumor was associated with the overall aggressiveness of her disease, are consistent with the clinical association between vimentin expression and ER negativity.

In summary, the LCC15-MB cells are shown to selectively re-colonize bone after arterial inoculation in the nude mouse model, and apparently have retained certain molecular traits responsible for this process. Thus, they provide an new resource with which to further define the molecular basis of bone metastasis, a painful and debilitating aspect of breast cancer.

Acknowledgements

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References

1. Parker SL, Tong T, Bolden S, Wingo PA. Cancer statistics, CA Cancer J Clin 1997; 47: 5–27.
2. Cadman E, Bertino JR. Chemotherapy of skeletal metastases. Int J Radiat Oncol Biol Phys 1976; 1: 1211–5.
3. Drews M, Dickson RB. Osseous complications of malignancy. In Lockich JJ (ed) Clinical Cancer Medicine; Treatment Tactics. Boston: G.K. Hall 1980; 97–125.
4. Enneking WF. Metastatic Carcinoma; Musculoskeletal Tumor Surgery. New York: Churchill Livingstone 1983.
5. Harrington KD. Orthopedic Management of Metastatic Bone Disease. St. Louis: Mosby 1988.
6. Wirth CR. Metastatic bone cancer. Curr Probl Cancer 1979; 3: 1–36.
7. Orr FW, Kostenuik P, Sanchez-Sweatman OH, Singh G. Mechanisms involved in the metastasis of cancer to bone. Breast Cancer Res Treat 1993; 25: 151–63.
8. Orr FW, Varani J, Gondek MD et al. Partial characterization of a bone-derived chemotactic factor for tumor cells. Am J Pathol 1980; 99: 43–52.
9. Diel IJ, Kaufmann M, Costa SD et al. Micrometastatic breast cancer cells in bone marrow at primary surgery: prognostic value in comparison with nodal status. J Natl Cancer Inst 1996; 88: 1652–8.
10. Yoneda T, Sasaki A, Mundy GR. Osteolytic bone metastasis in breast cancer. Breast Cancer Res Treat 1994; 32: 73–84.
11. Price JE, Polyzos A, Zhang RD, Daniels LM. Tumorigenicity and metastasis of human breast carcinoma cell lines in nude mice. Cancer Res 1990; 50: 717–21.
12. Price JE, Zhang RD. Studies of human breast cancer metastasis using nude mice. Cancer Metastasis Rev 1990; 8: 285–97.
13. Arguello F, Baggs RB, Frantz CN. A murine model of experimental metastasis to bone and bone marrow. Cancer Res 1988; 48: 6876–81.
14. Nakai M, Mundy GR, Williams PJ et al. A synthetic antagonist to laminin inhibits the formation of osteolytic metastases by human melanoma cells in nude mice. Cancer Res 1992; 52: 5395–9.
15. Sung V, Cattell DA, Bueno J et al. Human breast cancer cell metastasis to long bone and soft organs of nude mice: A quantitative assay. Clin Exp Metastasis 1997; 15: 173–83.
16. Mbalaviele G, Dunstan CR, Sasaki A et al. E-cadherin expression in human breast cancer cells suppresses the development of osteolytic bone metastases in an experimental metastasis model. Cancer Res 1996; 56: 4063–70.
17. Sasaki A, Boyce BF, Story B et al. Bisphosphonate risedronate reduces metastatic human breast cancer burden in bone in nude mice. Cancer Res 1995; 55: 3551–7.
18. Evans CE, Ward C, Braidman IP. Breast carcinomas synthesize factors which influence osteoblast-like cells independently of osteoclasts in vitro. J Endocrinol 1991; 128: R5–8.
19. Clohisy DR, Ogilvie CM, Ramnaraine ML. Tumor osteolysis in osteopetrotic mice. J Orthop Res 1995; 13: 892–7.
20. Ohishi K, Fujita N, Morinaga Y, Tsuruo T. H-31 human breast cancer cells stimulate type I collagenase production in osteoblast-like cells and induce bone resorption. Clin Exp Metastasis 1995; 13: 287–95.
21. Mundy GR, Yoneda T. Facilitation and suppression of bone metastasis. Clin Orthop 1995; 312: 34–44.
22. Engel LW, Young NA, Tralka TS et al. Establishment and characterization of three new continuous cell lines derived from human breast carcinomas. Cancer Res 1978; 38: 3352–64.
23. Clarke R, Leonessa F, Brunner N et al. *In vitro* models of human breast cancer. In: Harris JR, Lippman ME, Morrow M, Hellman (eds) Diseases of the Breast. New York: Lippincott, J.B., Co. 1995; 245–261.
24. Engel LW, Young NA. Human breast carcinoma cells in continuous culture: A review. Cancer Res 1978; 38: 4327–39.
25. Cailleau R, Olive M, Cruciger QV. Long-term human breast carcinoma cell lines of metastatic origin: Preliminary characterization. *In Vitro* 1978; 14: 911–5.
26. Robertson JF. Oestrogen receptor: a stable phenotype in breast cancer. Br J Cancer 1996; 73: 5–12.
27. Kaufmann M. Review of known prognostic variables. Recent Results Cancer Res 1996; 140: 77–87.
28. Gilles C, Thompson EW. The epithelial to mesenchymal transition and metastatic progression in carcinoma. The Breast Journal 1996; 2: 83-96.
29. Kulesh DA, Oshima RG. Cloning of the human keratin 18 gene and its expression in nonepithelial mouse cells. Mol Cell Biol 1988; 8: 1540–50.
30. Noguchi S, Aihara T, Motomura K et al. Detection of breast cancer micrometastases in axillary lymph nodes by means of reverse transcriptase-polymerase chain reaction. Comparison between muc1 mRNA and keratin 19 mRNA amplification. Am J Pathol 1996; 148: 649–56.
31. Clarke R, Morwood J, van den Berg HW et al. Effect of cytotoxic drugs on estrogen receptor expression and response to tamoxifen in MCF-7 cells. Cancer Res 1986; 46: 6116–9.
32. Ferguson AT, Lapidus RG, Baylin SB, Davidson NE. Demethylation of the estrogen receptor gene in estrogen receptor-negative breast cancer cells can reactivate estrogen receptor gene expression. Cancer Res 1995; 55: 2279–83.
33. Thompson EW, Paik S, Brunner N et al. Association of increased basement membrane invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines. J Cell Physiol 1992; 150: 534–44.
34. Sommers CL, Thompson EW, Torri JA et al. Cell adhesion molecule uvomorulin expression in human breast cancer cell lines: Relationship to morphology and invasive capacities. Cell Growth Differ 1991; 2: 365–72.
35. Sommers CL, Heckford SE, Skerker JM et al. Loss of epithelial markers and acquisition of vimentin expression in adriamycin- and vinblastine-resistant human breast cancer cell lines. Cancer Res 1992; 52: 5190–7.
36. Sommers CL, Byers SW, Thompson EW et al. Differentiation state and invasiveness of human breast cancer cell lines. Breast Cancer Res Treat 1994; 31: 325–35.
37. Bae SN, Arand G, Azzam H et al. Molecular and cellular analysis of basement membrane invasion by human breast cancer cells in matrigel-based in vitro assays. Breast Cancer Res Treat 1993; 24: 241–55.
38. Traweek ST, Liu J, Battifora H. Keratin gene expression in non-epithelial tissues. detection with polymerase chain reaction. Am J Pathol 1993; 142: 1111–8.

39. Datta YH, Adams PT, Drobyski WR et al. Sensitive detection of occult breast cancer by the reverse-transcriptase polymerase chain reaction. J Clin Oncol 1994; 12: 475–82.
40. Robertson JF, Dixon AR, Nicholson RI et al. Confirmation of a prognostic index for patients with metastatic breast cancer treated by endocrine therapy. Breast Cancer Res Treat 1992; 22: 221–7.
41. Clark GM, Sledge GW, Jr., Osborne CK, McGuire WL. Survival from first recurrence: relative importance of prognostic factors in 1,015 breast cancer patients. J Clin Oncol 1987; 5: 55–61.
42. Koenders PG, Beex LV, Langens R et al. Steroid hormone receptor activity of primary human breast cancer and pattern of first metastasis. The breast cancer study group. Breast Cancer Res Treat 1991; 18: 27–32.
43. Mano H, Yuasa T, Kameda T et al. Mammalian mature osteoclasts as estrogen target cells. Biochem Biophys Res Commun 1996; 223: 637–42.
44. Hoshino S, Inoue S, Hosoi T et al. Demonstration of isoforms of the estrogen receptor in the bone tissues and in osteoblastic cells. Calcif Tissue Int 1995; 57: 466–8.
45. Shen V, Birchman R, Xu R et al. Effects of reciprocal treatment with estrogen and estrogen plus parathyroid hormone on bone structure and strength in ovariectomized rats. J Clin Invest 1995; 96: 2331–8.
46. Lindsay R. The oestrogen receptor in bone-evolution of our knowledge. Br J Obstet Gynaecol 1996; 103: Suppl 13, 16–8; discussion 18–9.
47. Dickson RB, Lippman ME. Growth factors in breast cancer. Endocr Rev 1995; 16: 559–89.
48. Allred DC, Clark GM, Molina R et al. Overexpression of Her-2/neu and its relationship with other prognostic factors change during the progression of *in situ* to invasive breast cancer. Hum Pathol 1992; 23: 974–9.
49. Allred DC, Clark GM, Tandon AK et al. Her-2/neu in node-negative breast cancer: Prognostic significance of overexpression influenced by the presence of in situ carcinoma. J Clin Oncol 1992; 10: 599–605.
50. Domagala W, Lasota J, Dukowicz A et al. Vimentin expression appears to be associated with poor prognosis in node-negative ductal NOS breast carcinomas. Am J Pathol 1990; 137: 1299–304.
51. Domagala W, Lasota J, Bartkowiak J et al. Vimentin is preferentially expressed in human breast carcinomas with low estrogen receptor and high Ki-67 growth fraction. Am J Pathol 1990; 136: 219–27.
52. Gilles C, Polette M, Piette J et al. Vimentin expression in cervical carcinomas:association with the invasive and the migratory phenotype of tumor cells. J Pathol 1996; 180: 175–180.
53. Savagner P, Boyer B, Valles AM et al. Modulations of the epithelial phenotype during embryogenesis and cancer progression. Cancer Treat Res 1994; 71: 229–49.
54. Iwaya K, Thompson EW, Azumi N. Vimentin expression is a predictor of early recurrence and death in node negative carcinoma patients [Abstract]. Mod Pathol 1995; 8: 19A.

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CANCER LETTERS

Differential distribution of protein phosphatase 2A in human breast carcinoma cell lines and its relation to estrogen receptor status

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Abstract

Protein phosphatase 2A (PP2A) acts as a growth suppresser and is negatively influenced by oncogenic signals. We determined its activity in various human breast carcinoma (HBC) cell types to understand its relationship to estrogen receptor (ER) expression as well as to the distribution of protein kinase C (PKC), an opposing enzyme. PP2A activity was measured using a preferred substrate, histone H1 phosphorylated by PKC. PP2A activity was higher in both the soluble and nuclear fractions of ER-positive cell lines (MCF-7, T47D and ZR-75-1) than in the ER-negative cell lines (MDA-MB-231, Hs578T and BT-20). PP2A multiple forms ($2A_0$, $2A_1$, $2A_2$), separated by DEAE–cellulose chromatography and immunoblot analysis of PP2A catalytic subunit, also showed similar differences in these two HBC cell types. In all cases, PP2A distribution was inversely correlated with the PKC activity profile. Moreover, PP2A activity in MCF-7 cells maintained in estrogen-depleted medium was low. Nonetheless, it was induced by a prolonged treatment with 17β-estradiol, this induction being blocked by the antiestrogens, tamoxifen and ICI-182,780. Studies in both MCF-7 transfectants stably overexpressing *ras* and MDA-MB-231 transfectants stably expressing ER, suggested that a low PP2A distribution in ER-negative HBC cell types may be related to tumor progression rather than the loss of ER. Conceivably, the presence of high PP2A along with low PKC in ER-positive HBC cell types may be related to the restricted cell growth associated with the retention of a certain degree of differentiation or hormonal control. Conversely, the presence of low PP2A along with high PKC in ER-negative cell types may be related to hormone-independent enhanced cell growth.

Keywords: Protein phosphatase 2A; Protein kinase C; Estrogen receptor; Breast carcinoma cells; Nuclear localization; Tumor progression

1. Introduction

Breast carcinoma cell growth is influenced by an interplay between steroidal hormones and autocrine as well as paracrine growth factors [1,2]. The importance of protein kinase C (PKC) in the expression and function of estrogen receptor (ER) and epidermal growth factor (EGF) family peptides has been emphasized [2]. Previous studies have shown that ER-positive human breast carcinoma (HBC) cell types possess a lower activity of both PKC and EGF-R expression compared to ER-negative cell types [3]. However, understanding the state of phosphorylation of these receptors by following the activation of protein kinases alone is often difficult, since these events also are equally influenced by the negative regulation involving protein phosphatases.

Protein phosphatase 2A (PP2A) may preferentially

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dephosphorylate some protein substrates that are phosphorylated by PKC [4,5]. In a manner similar to phorbol esters and some oxidants, which elicit cellular responses and tumor promotion by directly activating PKC [6,7], tumor promoters such as okadaic acid and some oxidants also can elicit similar effects by directly inhibiting PP2A [8,9]. PP2A is considered to act as a 'growth suppressor' and its activity may be negatively regulated by oncogenic signals [10]. Viral oncogenic protein small-t antigen stimulates cell growth by inhibiting PP2A activity [11]. Furthermore, PP2A activity is decreased by phosphorylation at tyrosine residues by non-receptor- and receptor-linked tyrosine protein kinases including EGF receptor and insulin receptor [10]. Given the importance of EGF receptor and the related HER-2/*neu* in breast tumor growth, the negative modulation of PP2A may affect HBC cell growth regulation.

PP2A exists in the cell as a heterotrimeric complex consisting of a 36 kDa catalytic C subunit, 65 kDa structural/regulatory A subunit, and a variable regulatory B subunit [4,12]. The variable B subunits have been shown to contain target information that directs the PP2A to distinct intracellular locations [4,12]. The variable regulatory subunits, B56δ, B56γ3, and B56γ1 have been shown to localize PP2A to the nucleus [13]. Since okadaic acid has been shown to influence ER down-regulation via inhibition of PP2A [14], there is a possibility that besides regulation by various protein kinases [2], ER in the nucleus may also be regulated by PP2A. However, there are no reports on the relative distribution of PP2A in various HBC cell types in relation to either ER or protein kinases such as PKC.

In this study, we show higher levels of PP2A both in the soluble and nuclear fractions of ER-positive HBC cell lines compared to ER-negative HBC cell lines. This PP2A distribution profile is inversely correlated with the level of PKC activity in both types of cell lines and is related to ER status and tumor progression.

2. Materials and methods

2.1. Materials

The catalytic subunit of PKA from porcine heart, leupeptin, and pepstatin A were obtained from the Sigma Chemical Co. [γ-^{32}P]ATP (specific activity, 20 Ci/mmol) was purchased from ICN Radiochemicals. Rabbit polyclonal antibodies to the C-terminal region of human PP2A (residues 296–309) were obtained from Upstate Biotechnology. Microcystin-LR and okadaic acid were obtained from the LC Services Corporation. Calf thymus histone H1 was phosphorylated by PKC in the presence of Ca^{2+}, phosphatidylserine, and diolein [5]. This substrate phosphoprotein was referred to as 'histone H1(C)'. A catalytic subunit of PKA was used to phosphorylate histone H1 as described by others [15] and this substrate was referred to as 'histone H1(A)'. Various HBC cell lines were obtained from the American Type Culture Collection. A stably transfected MCF-7 cell line overexpressing *ras* was previously reported [16]. An MDA-MB-231 cell line stably expressing ER was established by transfection of the parent cell line with ER-α cDNA as previously reported [17].

2.2. Preparations of cell extracts

HBC cells were grown in 100-mm Petri dishes in minimal essential medium (MEM) supplemented with 5% fetal calf serum. Confluent cells (10 to 15×10^6) were homogenized in 3.5 ml of buffer A (20 mM Tris–HCl, pH 7.5/1 mM EDTA/1 mM dithiothreitol/0.5 mM phenylmethylsulfonyl fluoride/1 μM leupeptin/0.15 μM pepstatin A). The homogenates were centrifuged at $1000 \times g$ for 10 min and the supernatant was referred to as the soluble fraction.

2.3. Isolation of nuclear fraction

Cells were homogenized in 50 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (pH 6.5), 1 mM $CaCl_2$, 0.5 M hexylene glycol, and 1 μM leupeptin. Crude nuclei were isolated by centrifugation at $1000 \times g$ for 3 min. The nuclear pellet was resuspended in 3 ml of suspension buffer (50 mM Tris–HCl, pH 7.5) 0.3 mM sucrose, 25 mM KCl, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin and centrifuged at $10\,000 \times g$ for 10 min. The purified nuclear pellet was extracted with buffer A containing 1% NP-40.

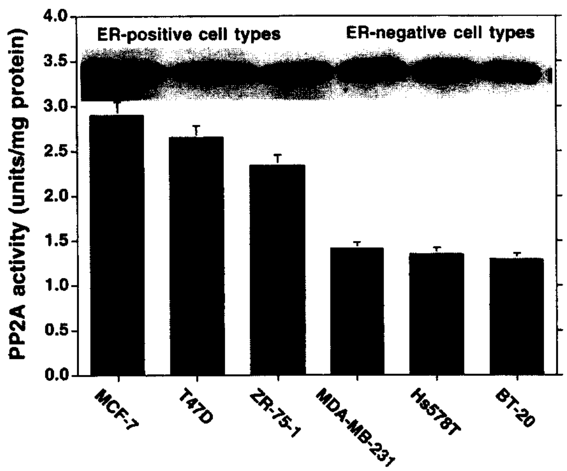

Fig. 1. The enzyme activity and immunoblot of PP2A from the soluble fractions of ER-positive and ER-negative HBC cell lines. PP2A activity was directly determined in the diluted soluble fractions. Protein (40 μg) was subjected to SDS–polyacrylamide electrophoresis and then to immunoblotting as described in Section 2. The 36 kDa immunoreactive PP2A catalytic subunit from the soluble fraction is presented at the top of the histogram bar representing the corresponding PP2A activity present in that fraction of the indicated cell type. The values for PP2A activity represent mean and SEM of three different experiments. There was a statistically significant difference between the ER-positive and ER-negative groups of cell types ($P<0.0001$).

2.4. DEAE–cellulose chromatographic separation of PP2A multiple forms and PKC

The cell extracts (1.2 mg protein) were applied to 1-ml DEAE–cellulose (DE-52) columns previously equilibrated with buffer B (20 mM Tris–HCl, pH 7.5/1 mM EDTA/1 mM dithiothreitol). After washing the columns with 4 ml of buffer B, the bound proteins were step-wise eluted with 2.5 ml of 0.1 M, 0.15 M, 0.2 M and 0.4 M NaCl in buffer B and these four peaks were designated as peaks I, II, III, and IV, respectively (Fig. 1). PKC activity was eluted with 0.1 M NaCl (peak I). The PP2A activity was eluted in peaks II, III, IV.

2.5. PP2A assays

The assay was carried out using the multiwell filtration approach as previously described [18]. Briefly, 10–20 μl of diluted cell extracts were pipetted in triplicates in 96-well microtiter plates. The reaction was initiated by the addition of 50 μl of a ^{32}P-labeled H1 histone mixture containing 50 mM Tris–HCl, pH 7.5/1 mM EDTA/1 mM DTT/0.1 M NaCl/0.2 mg per ml bovine serum albumin/1 μM leupeptin/0.3 μM pepstatin A/75 pmol of ^{32}P-labeled histone H1(C). After incubation at 30°C for 10 min, the reaction was terminated by adding 75 μl of 70% trichloroacetic acid and the samples were filtered. The ^{32}Pi radioactivity present in 75 μl of the filtrate was counted. The PP2A activity was obtained by subtracting the control activity observed in the presence of microcystin-LR (0.5 nM) from the total activity observed without the inhibitor. One unit of PP2A liberates one nmol of phosphate from histone H1(C) per min at 30°C.

2.6. PKC assay

Briefly, PKC reaction samples containing 20 mM Tris–HCl, pH 7.5/10 mM $MgCl_2$/0.33 mM $CaCl_2$/0.1 mM [γ-^{32}P]ATP/histone H1 (0.1 mg/ml), and 25 μl of PKC sample (peak I) in a total volume of 125 μl were incubated at 30°C for 5 min in 96-well plates with fitted filtration discs [19]. The radioactivity incorporated into histone H1 was counted. One unit of PKC transfers 1 nmol of phosphate to histone H1 per min at 30°C.

2.7. Immunoblotting of PP2A catalytic subunit

The soluble and nuclear fraction of various HBC cell types were electrophoresed on 12% SDS–polyacrylamide gels and the proteins were transferred to polyvinylidene difluoride membrane. The catalytic subunit was detected by using polyclonal antibodies that recognize the C-terminal region of the PP2A catalytic subunit. Immunoreactive bands were visualized by utilizing alkaline phosphatase-conjugated second antibody (Bio-Rad) and CDP-Star chemiluminescence reagents (New England Nuclear).

2.8. 17β-Estradiol treatment of MCF-7 cells

Initially, MCF-7 or T47D cells were grown to confluence in MEM-medium supplemented with 5% fetal calf serum. The medium was then replaced with an estrogen-free medium (phenol red-free MEM

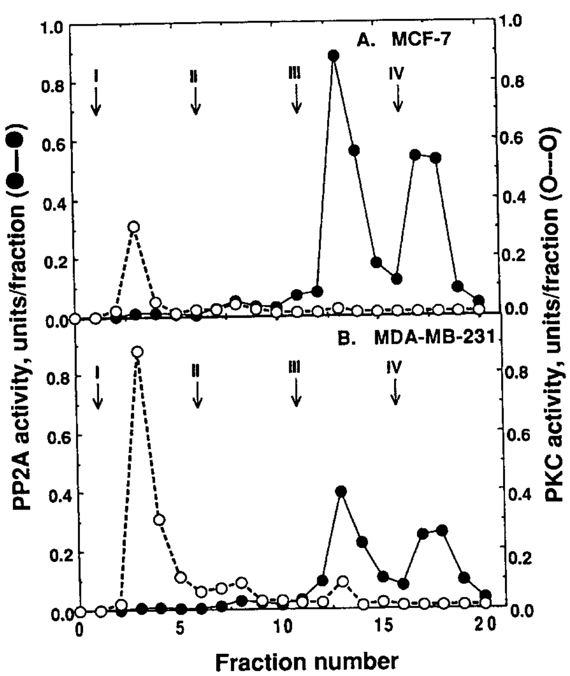

Fig. 2. DEAE–cellulose chromatography of PP2A multiple forms and PKC from MCF-7 and MDA-MB-231 cells. The soluble fraction was applied to 1-ml DEAE–cellulose (DE-52) columns previously equilibrated with buffer C. After washing the columns with 4 ml of buffer C, the bound PP2A isoforms were step-wise eluted as indicated by arrows (I, II, III, IV) with 2.5 ml of 0.1 M, 0.15 M, 0.2 M, 0.4 M NaCl in buffer C. Fractions of 0.5 ml were collected; the activities of PP2A and PKC were determined.

medium supplemented with 5% dextran-coated charcoal-treated serum) and left for 1 week. These cells were seeded in 100-mm Petri dishes at a low cell density and maintained in an estrogen-free medium supplemented with either 17β-estradiol (10 nM) alone or in combination with antiestrogens, tamoxifen (100 nM) or ICI-182,780 (100 nM).

2.9. Statistical analysis

PP2A and PKC activities in various cell types were analyzed by one-way ANOVA followed by Scheffe's multiple range test to separate means. A paired *t*-test was used to determine differences in activity of PP2A with estrogen and antiestrogen treatments. Significance was set at $P<0.05$.

3. Results

3.1. Use of PKC-phosphorylated histone H1 as a substrate for PP2A assay

Histone H1 has multiple sites for phosphorylation. Depending on the site that is phosphorylated, it is dephosphorylated by different protein phosphatases [5]. Histone H1, phosphorylated at serine 103 by PKC, is preferentially dephosphorylated by PP2A, while histone H1, phosphorylated at the N-terminal site by protein kinase A or other protein kinases, is dephosphorylated by PP1 [5]. One study suggested that the physiological histone H1 phosphatase is PP2A [20], whereas another study showed that it is PP1 instead of PP2A [21]. Such discrepancy may be due to the difference in protein kinase that phosphorylates histone H1. However, in the current study, histone H1 phosphorylated by PKC was used as the substrate for PP2A not only for achieving the specificity for PP2A assay [5], but also to compare with PKC activity which employed histone H1 as the substrate. Further specificity for PP2A assay was achieved by using the control with microcystin-LR at a low concentration (0.5 nM), sufficient to completely inhibit the activity of PP2A but not PP1 [22].

Since the association of various B56 regulatory subunits can alter the substrate specificity of PP2A, we initially tested whether or not the PP2A present in various HBC cell types preferentially dephosphorylated histone H1(C). Nearly 95% of the dephosphorylation of histone H1(C) by various HBC cell extracts was inhibited by either 2 nM okadaic acid or 0.5 nM microcystin-LR (data not shown). This suggested that most of the observed phosphatase activity using this substrate was contributed by PP2A. The PP2A dephosphorylating activity towards histone H1(A) was only 5% of that observed with histone H1(C) in all cell types tested, suggesting that PP2A may be closely related to the PKC pathway.

3.2. Relative distribution of PP2A in soluble fractions of various HBC cell types

As shown in Fig. 1, PP2A activity was higher in the soluble fractions of all three ER-positive cell lines (MCF-7, T47D, ZR-75-1) compared to all three ER-negative cell lines (MDA-MB-231, Hs578T, BT-20)

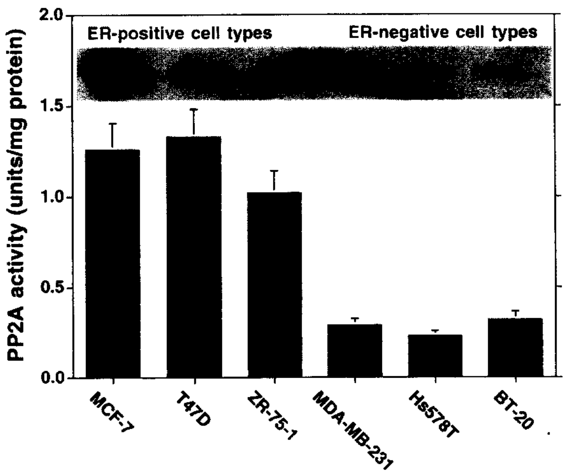

Fig. 3. The enzyme activity and immunoblot of PP2A from the nuclear fractions of ER-positive and ER-negative HBC cell lines. The PP2A activity is presented as a bar of the histogram and the corresponding immunoreactive protein of the catalytic subunit of PP2A is presented at the top of the bar for each cell type. The values for PP2A activity represent mean and SEM of three different experiments. There was a statistically significant difference between the ER-positive and ER-negative groups of cell types ($P<0.0001$).

tested ($P<0.001$). Furthermore, immunoblot analysis showed higher amounts of PP2A catalytic subunit in ER-positive cell types compared to that present in ER-negative cell types, correlating with the PP2A activity determinations (Fig. 1).

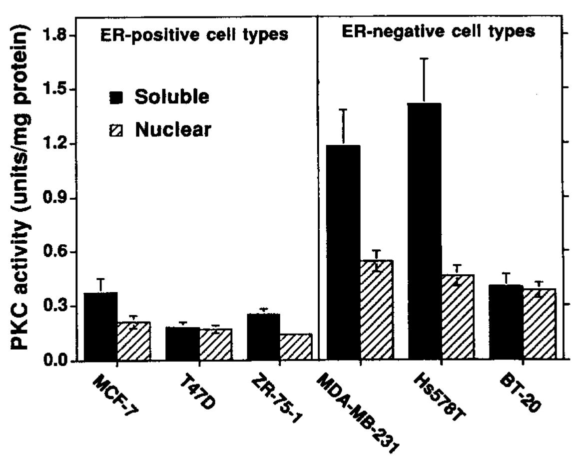

Fig. 4. PKC activity in the soluble and nuclear fractions of ER-positive and ER-negative HBC cell types. PKC activity was determined after subjecting the soluble and nuclear fractions to DEAE–cellulose chromatography. The enzyme activity was expressed in units comparable to the units of PP2A expressed in Figs. 1 and 3.

We further determined whether the observed difference in the activity of PP2A in the soluble fractions of these two HBC cell types might have been caused by differences in the distribution of activators/inactivators of PP2A or by an alteration in the regulatory subunits which can influence the substrate specificity of this enzyme. Previous studies showed that PP2A activity from tissues can be resolved by DEAE–cellulose chromatography into three forms that are designated as $PP2A_0$, $PP2A_1$ and $PP2A_2$ [23]. All these isoforms of PP2A have the same catalytic subunit C, while there are regulatory subunits A and B' for $PP2A_0$, regulatory subunits A and B for $PP2A_1$ and only the regulatory subunit A for $PP2A_2$ [23]. As shown in Fig. 2, PP2A activity from the soluble fraction of MCF-7 and MDA-MB-231 cells were eluted into three peaks. A comparison with the previously established procedure [23] identified PP2A activity eluted in peaks II, III, and IV as $PP2A_0$, $PP2A_1$ and $PP2A_2$, respectively. The ER-positive cells exhibited a higher activity of PP2A multiple forms than that of ER-negative cell lines. Therefore, the observed differences were not contributed by activators/inactivators or the change in subunit composition of PP2A.

3.3. Nuclear association of PP2A in various HBC cell types

As shown in Fig. 3, the PP2A activity associated with the nuclear fraction was 3- to 6-fold higher in the ER-positive cell types compared to that in the ER-negative cell types ($P<0.001$). The difference between these two HBC cell types was more pronounced in the nuclear fraction than that observed in the soluble fraction. The immunoblot analysis also revealed higher levels of the PP2A catalytic subunit corresponding to the higher activity of PP2A in the nuclear fraction of ER-positive cell types (Fig. 3).

3.4. Relative distribution of PKC in various HBC cell types

As shown in Fig. 4, PKC activity was lower in the soluble fraction of ER-positive cell lines than ER-negative cell lines ($P<0.001$). These data was in agreement with the previously reported results [3]. Furthermore, in the current study, this difference in

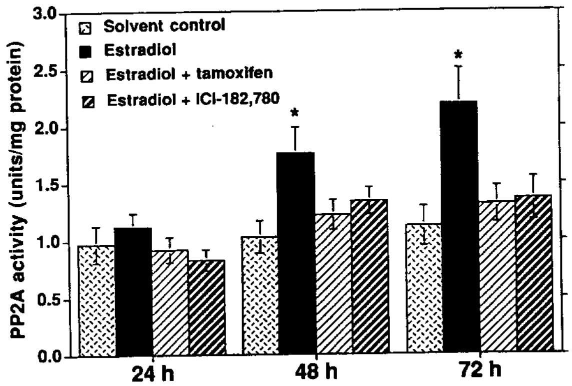

Fig. 5. Induction of PP2A by 17β-estradiol treatment in MCF-7 cells and its prevention by antiestrogens. MCF-7 cells were maintained initially in estrogen-depleted medium. Petri dishes were divided into four sets; one set was treated with ethanol as a solvent control; the second set was treated with 17β-estradiol (10 nM) and the other two sets were treated with estradiol along with 100 nM antiestrogens, tamoxifen or ICI-182,780. At indicated periods of time, PP2A activity present in the soluble fraction was determined in all four sets. *Significantly different from respective solvent control ($P<0.05$) by paired t-test.

PKC activity was also observed in the nuclear fraction. The current study further revealed an inverse relationship in the distribution of activities of PP2A (Figs. 1 and 3) and PKC in both the soluble and nuclear fractions with correlation coefficients of −0.584, −0.798, respectively.

3.5. Induction of PP2A by estrogen in ER-positive HBC cell types

In MCF-7 cells maintained in estrogen-depleted medium, PP2A activity decreased in the cytosol. Upon treatment with 17β-estradiol, there was a time-dependent increase in PP2A activity in these cells (Fig. 5). The activity increase became apparent only after 24h. Immunoreactive staining of the PP2A catalytic subunit also increased with 17β-estradiol treatment in MCF-7 cells (data not shown). Both the non-steroidal antiestrogen tamoxifen and the steroidal antiestrogen, ICI-182,780 abolished the induction of PP2A activity by estrogen (Fig. 5). In the nuclear fraction, similar results also were obtained (data not shown). For example, in the 48-h control without estrogen, PP2A activity was 0.66 units/mg protein; however, in the presence of estrogen, this increased to 1.18 units/mg protein. In all cases, immunoblot analysis of PP2A was in agreement with its activity. A similar type of induction of PP2A by estrogen was observed in T47D cells.

3.6. Distribution of PP2A in ras- and ER-transfected HBC cell types

A lower activity of PP2A in ER-negative cell types, whether it is related to tumor progression or the loss of ER, was determined by comparing the PP2A and PKC activity profile in MCF-7 cells progressed by *ras* transfection and in MDA-MB-231 cells transfected with ER. Although the PP2A activity in the soluble fraction was lower in the *ras*-transfected cells than the parent MCF-7 cells, this was found to be not statistically significant. However, this activity in nuclear fraction was significantly lower ($P<0.05$) in *ras*-transfected cells than that in the parent cell type (Table 1). The PP2A/PKC activity profile in MCF-7 *ras*-transfected cells more closely resembled that observed with ER-negative cell types. In MDA-MB-231 transfectants stably expressing ER, there was only

Table 1
PP2A and PKC activities in *ras*-transfected MCF-7 and ER-transfected MDA-MB-231 HBC cells[a]

Cell type	Soluble fraction		Nuclear fraction	
	PP2A (units/mg protein)	PKC (units/mg protein)	PP2A (units/mg protein)	PKC (units/mg protein)
MCF-7 (wild type)	2.61 ± 0.34	0.45 ± 0.03	1.21 ± 0.09	0.28 ± 0.04
ras-transfected MCF-7	1.67 ± 0.14	0.78 ± 0.09	0.58 ± 0.07*	0.43 ± 0.05
MDA-MB-231 (wild type)	1.24 ± 0.16*	1.23 ± 0.15*	0.36 ± 0.05*	0.51 ± 0.07
ER-transfected MDA-MB-231	1.42 ± 0.17*	1.02 ± 0.13*	0.42 ± 0.06*	0.47 ± 0.06

[a] Activities are expressed in units/mg protein. Values represent the mean and SEM of three experiments. Values significantly different from wild type MCF-7 are marked with an asterisk (*$P<0.05$).

a small difference in the PP2A/PKC activity profile compared to the parent cell type lacking ER.

4. Discussion

Higher levels of PP2A were observed in both soluble and nuclear fractions of ER-positive cell lines compared to ER-negative cell lines, inversely correlating with the PKC activity profile. PP2A has been implicated in the regulation of CREB, AP-1, p53, and telomerase [24–27]. PP2A was reported to play a role in the regulation of the cell cycle [24]. It has been shown to be involved in suppressing the activation of cell-cycle regulating protein kinase $p34^{cdc2}$ by increasing its inhibitory phosphorylation [28,29], as well as in the dephosphorylation of its protein substrates [20]. Furthermore, PP2A also has been shown to dephosphorylate and inactivate cdc25C, a protein phosphatase involved in the activation of $p34^{cdc2}$ [30]. Therefore, a constitutive high expression of PP2A in ER-positive HBC cell types may enhance the inactivation of cell cycle-associated $p34^{cdc2}$ and cdc25C by affecting their phosphorylation. Nevertheless, further studies are certainly required to understand the role of PP2A in the negative regulation of growth of these cell types.

In the ER-positive MCF-7 and T47D cells, depletion of estrogen from the medium decreased the levels of PP2A catalytic subunit. Replenishment of estrogen resulted in increased levels of PP2A with a lag period of more than 24 h. The antiestrogens TAM and ICI-182,780 prevented the induction of PP2A, suggesting the involvement of ER-regulated events. However, previous studies did not reveal a classical estrogen-responsive element in the promoter region of the gene that encodes the PP2A catalytic subunit [31]. The slow induction by estradiol would be consistent with a secondary response rather than a primary response to estradiol as recently discussed for other genes [32]. A mere presence of low PP2A activity may not lead to growth stimulation as seen in the estrogen-depleted MCF-7 cells. Certainly, additional estrogen-regulated components may be required for growth stimulation. However, PP2A induced as a secondary response to estrogen may slow down the cell growth and this feedback mechanism might be lacking in the ER-negative cell types.

The presence of a low activity of PP2A and a high activity of PKC in *ras*-transfected cell types, especially in the nuclear fraction, may be related to tumor progression. Overexpression of v-*ras* was shown to induce estrogen-independent growth of MCF-7 cells in nude mice [33]. Previous studies showed higher levels of PKC and lower levels of PP2A in drug-resistant MCF-7 cells compared to the parent MCF-7 cell line [34]. In MDA-MB-231 transfectants stably expressing ER, there was no increase in levels of PP2A compared to the wild type MDA-MB-231 cell line lacking ER. This suggests that the presence of ER alone may not lead to an increased expression of PP2A.

BT-20 cells were originally classified as ER-negative based on radio-ligand binding analysis. However, subsequent studies showed that this cell type has a truncated form of ER lacking the hormone-binding domain but possessing constitutive transcriptional regulatory activity [35]. Recently, a new type of ER (ER-β) has been shown to be present in MCF-7 and T47D cell lines [36]. A variant of ER-β devoid of hormone-binding domain has been shown in MDA-MB-231 cells [36]. Thus, higher levels of PP2A may be present in HBC cell types having hormone-responsive ER, which may be related to the retention of some degree of differentiation, while low levels of PP2A is present in HBC cell types having hormone-unresponsive ER variants, which may be related to tumor progression.

PP2A is considered to be a growth suppressor, while protein kinases such as PKC and mitogen-activated protein (MAP) kinases are growth supporters. Several of these growth-supporting kinases are phosphoproteins in their activated forms. For example, PKC and MAP kinases lose their activity upon dephosphorylation by PP2A [11,37]. Cellular stresses such as oxidants and okadaic acid are not only known to inhibit PP2A but also directly or indirectly activate PKC and other protein kinases such as MAP kinases [7,9,38,39]. Therefore, an inhibition of PP2A maintains not only the protein substrates in the hyperphosphorylated state but also protein kinases in a sustained activated form. Besides a negative modulation of PP2A by post-translational modifications, a constitutive low expression of PP2A in tumor cells may give an additional growth advantage. Conceivably, in ER-positive cells, the presence of high

activity of PP2A along with a low activity of PKC may be well suited for a low cell growth rate related to the retention of some degree of differentiation or hormonal control. Conversely, in ER-negative cell types, the low expression of PP2A along with high levels of PKC may have a role in supporting enhanced growth response to autocrine growth factors.

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References

[1] R.B. Dickson, M.E. Lippman, Growth factors in breast cancer, Endocrinol. Rev. 16 (1995) 559–589.

[2] I. Martinez-Lacaci, R.B. Dickson, Dual regulation of the epidermal growth factor family of growth factors in breast cancer by sex steroids and protein kinase C, J. Steroid Biochem. Mol. Biol. 57 (1996) 1–11.

[3] D. Fabro, W. Kung, W. Roos, R. Regazzi, U. Eppenberger, Epidermal growth factor binding and protein kinase C activities in human breast cancer cell lines: possible quantitative relationship, Cancer Res. 46 (1986) 2720–2725.

[4] P. Cohen, The structure and regulation of protein phosphatases, Annu. Rev. Biochem. 58 (1989) 453–508.

[5] S. Jakes, K.K. Schlender, Histone H1 phosphorylated by protein kinase C is a selective substrate for the assay of protein phosphatase 2A in the presence of phosphatase 1, Biochim. Biophys. Acta 967 (1988) 11–16.

[6] M. Castagna, Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, Y. Nishizuka, Direct activation of Ca^{2+}-activated phospholipid-dependent protein kinase by tumor promoting phorbol esters, J. Biol. Chem. 257 (1982) 7847–7851.

[7] R. Gopalakrishna, W.B. Anderson, Ca^{2+}- and phospholipid-independent activation of protein kinase C by selective oxidative modification of the regulatory domain, Proc. Natl. Acad. Sci. USA 86 (1989) 6758–6762.

[8] M. Suganuma, H. Fujiki, H. Suguri, S. Yoshizawa, M. Horita, M. Nakayasu, M. Ojika, K. Wakamatsu, K. Yamada, T. Sugimura, Okadaic acid: an additional non phorbol-12-tetradecanoate-13-acetate type tumor promoter, Proc. Natl. Acad.. Sci. USA 85 (1988) 1768–1771.

[9] R. Nemani, E.Y.C. Lee, Reactivity of sulfhydryl groups of the catalytic subunits of rabbit skeletal muscle protein phosphatases 1 and 2A, Arch. Biochem. Biophys. 300 (1993) 24–29.

[10] J. Chen, S. Parasons, D.L. Brautigan, Tyrosine phosphorylation of protein phosphatase 2A in response to growth stimulation and v-*src* transformation of fibroblasts, J. Biol. Chem. 269 (1994) 7957–7982.

[11] E. Sontag, S. Pedrov, C. Kamibayashi, D. Robbins, M. Cobb, M. Mumby, The interaction of SV40 small tumor antigen with protein phosphatase 2A stimulates the MAP kinase pathway and induces cell proliferation, Cell 75 (1993) 887–897.

[12] M.C. Mumby, G. Walter, Protein serine/threonine phosphatases: structure, regulation and function in cell growth, Physiol. Rev. 73 (1993) 673–699.

[13] B. McCright, A.M. Rivers, S. Audlin, D.M. Virshup, The B56 family of protein phosphatase 2A regulatory subunits encodes differentiation-induced phosphoproteins that target PP2A to both nucleus and cytoplasm, J. Biol. Chem. 271 (1996) 22081–22089.

[14] M. Borras, L. Hardy, F. Lempereur, A.H. el-Khissiin, N. Legros, R. Gol-Winkler, G. Leclercg, Estradiol-induced down-regulation of estrogen receptor. Effect of various modulators of protein synthesis and expression, J. Steroid Biochem. Mol. Biol. 48 (1994) 325–336.

[15] S.D. Killiea, J.H. Aylward, R.L. Mellgren, E.Y.C. Lee, Purification and properties of bovine myocardial phosphorylase phosphatase (protein phosphatase C), Arch. Biochem. Biophys. 191 (1978) 638–646.

[16] R. Clarke, N. Brunner, B.S. Katzenellenbogen, E.W. Thompson, M.J. Norman, C. Koppi, S. Paik, M.E. Lippman, R.B. Dickson, Progression of human breast cancer cells from hormone-dependent to hormone-independent growth both *in vitro* and *in vivo*, Proc. Natl. Acad. Sci. USA 86 (1989) 3649–3653.

[17] M.S. Sheikh, Z.-M. Shao, J-C. Chen, X-S. Li, A. Hussain, J.A. Fontana, Expression of estrogen receptors in estrogen receptor-negative human breast carcinoma cells: modulation of epidermal growth factor-receptor (EGF-R) and transforming growth factor α (TGFα) gene expression, J. Cell. Biochem. 54 (1994) 289–298.

[18] R. Gopalakrishna, U. Gundimeda, J.C. Wilson, Z.H. Chen, Multiwell filtration for rapid determination of protein phosphatase activity, Anal. Biochem. 212 (1993) 296–299.

[19] R. Gopalakrishna, Z.H. Chen, U. Gundimeda, J.C. Wilson, W.B. Anderson, Rapid filtration assays for protein kinase C and phorbol ester binding using multiwell plates with fitted filtration discs, Anal. Biochem. 206 (1992) 24–35.

[20] P. Ferrigano, T.A. Langan, P. Cohen, Protein phosphatase $2A_1$ is the major enzyme in vertebrate cell extracts that dephosphorylates several physiological substrates for cyclin-dependent protein kinases, Mol. Biol. Cell 4 (1993) 669–677.

[21] J.R. Paulson, J.S. Patzlaff, A.J. Vallis, Evidence that the endogenous histone H1 phosphatase in HeLa mitotic chromosomes is protein phosphatase 1, not protein phosphatase 2A, J. Cell Sci. 109 (1996) 1437–1447.

[22] R.E. Honkanen, J. Zwiller, R.E. Moore, S.L. Daily, B.S. Khatra, M. Dukelow, A.L. Boynton, Characterization of microcystin-LR, a potent inhibitor of type I and type 2A protein phosphatases, J. Biol. Chem. 265 (1990) 19401–19404.

[23] H.Y.L. Tung, S. Alemany, P. Cohen, Purification, subunit structure and properties of protein phosphatases $2A_0$, $2A_1$,

and $2A_2$ from rabbit skeletal muscle, Eur. J. Biochem. 148 (1985) 253–263.

[24] P. Turowski, A. Fernandez, B. Favre, N.J.C. Lamb, B.A. Hemmings, Differential methylation and altered conformation of cytoplasmic and nuclear forms of protein phosphatase 2A during cell cycle progression, J. Cell. Biol. 129 (1995) 397–410.

[25] A.S. Alberts, T. Deng, A. Lin, J.L. Meinkoh, A. Schonthal, M.C. Mumby, M. Karin, J.R. Feramisco, Protein phosphatase 2A potentiates activity of tumor promoters containing AP-1 binding elements, Mol. Cell. Biol. 13 (1993) 2104–2112.

[26] B.E. Wadzinski, W.H. Wheat, S. Jaspers, L.F. Peruski, R.L. Lickteig, G.L. Johnson, D.J. Klemm, Nuclear protein phosphatase 2A dephosphorylates protein kinase A-phosphorylated CREB and regulates CREB transcriptional stimulation, Mol. Cell. Biol. 13 (1993) 2822–2834.

[27] H. Li, L. Zhao, J.W. Funder, J. Liu, Protein phosphatase 2A inhibits nuclear telomerase activity in human breast cancer cells, J. Biol. Chem. 272 (1997) 16729–16732.

[28] T.H. Lee, C. Turck, M.W. Kirschner, Inhibition of cdc2 activation by INH/PP2A, Mol. Biol. Cell 5 (1994) 323–338.

[29] H. Rime, C. Jessus, R. Ozon, Tyrosine phosphorylation of $p34^{cdc2}$ is regulated by protein phosphatase 2A in growing immature xenopus oocytes, Exp. Cell Res. 219 (1995) 29–38.

[30] R.A. Barratt, G. Kao, W.G. McKenna, J. Kuang, R.J. Muschel, The G_2 block induced by DNA damage: a caffeine-resistant component independent of cdc25C, MPM-2 phosphorylation, and H1 kinase activity, Cancer Res. 58 (1998) 2639–2645.

[31] Y. Khew-Goodall, R.E. Mayer, F. Maurer, S.R. Stone, B.A. Hemmings, Structure and transcriptional regulation of protein phosphatase 2A catalytic subunit genes, Biochemistry 30 (1991) 89–97.

[32] R. Clarke, N. Brunner, Acquired estrogen independence and antiestrogen resistance in breast cancer: estrogen receptor-driven phenotypes?, Trends Endocrinol. Metab. 7 (1996) 25–36.

[33] A. Kasid, M.E. Lippman, A.G. Papageorge, D.R. Lowy, E.P. Gelmann, Transfection of v-*ras* DNA into MCF-7 cell bypasses their dependence on estrogen for tumorigenecity, Science 228 (1985) 725–726.

[34] D.D. Ratnasinghe, J.M. Phang, G.C. Yeh, Differential expression of protein kinase C and protein phosphatases 1 and 2A in adriamycin resistant human breast cancer cells, Proc. Am. Assoc. Cancer Res. 36 (1995) 330–.

[35] C.G. Castles, S.A. Fuqua, D.M. Klotz, S.M. Hill, Expression of a constitutive active estrogen receptor variant in estrogen receptor-negative BT-20 human breast cancer cell line, Cancer Res. 53 (1993) 5934–5939.

[36] E.A. Vladusic, A.E. Hornby, F.K. Guerra-Vladusic, R. Lupu, Expression of estrogen receptor β messenger RNA variant in breast cancer, Cancer Res. 58 (1998) 210–214.

[37] E.M. Dutil, L.M. Keranen, A.A. DePaoli-Roach, A.C. Newton, *In vivo* regulation of protein kinase C by transphosphorylation followed by autophosphorylation, J. Biol. Chem. 269 (1994) 29359–29362.

[38] R. Gopalakrishna, Z.H. Chen, U. Gundimeda, Nonphorbol tumor promoters okadaic acid and calyculin-A induce membrane translocation of protein kinase C, Biochem. Biophys. Res. Commun. 189 (1992) 950–957.

[39] L. Fialkow, C.K. Chan, D. Rotin, S. Grinstein, G.P. Downey, Activation of mitogen-activated protein kinase signaling pathway in neutrophils: role of oxidants, J. Biol. Chem. 269 (1994) 31234–31242.

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Editorial

The leucocyte nadir, a predictor of chemotherapy efficacy?

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Among many challenges in chemotherapy of cancer diseases, overtreatment remains one of the greatest. A substantial proportion of patients, perhaps the majority, do not benefit from the chemotherapy that they are offered, either in the adjuvant or in the palliative situation. In the adjuvant situation this is particularly serious, as the same patients will often experience the side-effects following the therapy. For such patients, there is no immediate way to assess the value of therapy.

The response of cancer cells to chemotherapy can simplistically be illustrated as related to two basic, pharmacological characteristics. The first is the accessibility of the therapeutic principle to the cancer cell: the drug in its active form has to reach the target, the malignant cell, wherever it is located. The second is the inherent sensitivity in the target cells, the cancer cells, to the treatment principle. This sensitivity results from a combination of genetic predisposition and acquired resistance. The acquired resistance could be due to previous exposure to toxic substances or be a characteristic from malignant evolution. The sensitivity versus resistance, or the expression of this relation in a tumour cell population, seems to be related to cell proliferation.

If the sensitivity of tumour cells to therapeutic principles is a reflection of the genetic predisposition, this sensitivity would theoretically be the same in all cells in an individual. If, on a given schedule of chemotherapy, the leucocyte nadir is low, this could then indicate more adequate exposure of drug to the tumour cells (and all other cells) in that individual.

In addition to the individual sensitivity-based genetic predisposition, tumour responsiveness to specific drugs, such as anthracyclines and cisplatinum, is probably also related to genetic changes in the tumour cells. Examples are p53 mutations, which seem to indicate lack of response to CMF and doxorubicin (Aas et al, 1996) while the opposite is indicated for cisplatinum and paclitaxel (Hawkins et al, 1996).

The hypothesis of an individual genetic predisposition is tested by Poikonen et al (1994). In patients with primary, node-positive breast cancer and who were given CMF as adjuvant chemotherapy, they conclude that leucocyte nadir is associated with favourable distant disease-free interval and that it could be useful as a biological marker of chemotherapy efficacy. This is a follow-up and confirmation of similar work from the same group (Saarto et al, 1997). In this previous paper, based on results from 211 stage II and III breast cancer patients, it was concluded that a low leucocyte nadir after another, anthracycline-containing adjuvant regimen predicated both prolonged distant disease-free interval and overall survival. The interesting concept of a higher biological dose intensity connected with low leucocyte nadirs and good prognosis compared to high dose intensity due to lower sensitivity (higher leucocyte nadir) is discussed.

Pointing in the same direction are studies on the effect on outcome of dose reductions. In a well conducted, although retrospective, study Colleoni et al (1997) found that patients who had major dose reductions based on severe neutropenia experienced less benefit from the treatment.

If the results in the papers presented by the Finnish group (Saarto et al, 1997; Poikonen et al, 1999) are supported by further documentation, it would support the concept of tailored, individual dosing based on individual sensitivity, best characterized by the leucocyte nadir after the first challenges with chemotherapy. An unresponsive bone marrow after initial standard dose indicates that the treatment is not well tolerated, but too well tolerated. This hypothesis should be tested in a prospective, randomized trial where the patients in the control arm of the trial are given chemotherapy according to traditional conventions, while the patients in the experiment are given individualized, tailored doses of chemotherapy according to bone marrow (leucocyte nadir) response. Perhaps the Finnish investigators will do this?

REFERENCES

Aas T, Børressen A-L, Geisler S, et al (1996) Spesific p53 mutations are associated with de novo resistance to doxorubicine in breast cancer patients. *Nat Med* **2**: 811–814

Colleoni M, Pricer K, Catiglione-Gertsch M et al (1998) Dose-response effect of adjuvant cyclophosphamide, methotrexate, 5-fluorouracil (CMF) in node-positive breast cancer. *Eur J Cancer* **34**: 1693–1700

Hawkins D, Demers G and Galloway D (1996) Inactivation of p53 enhances sensitivity to multiple chemotherapeutic agents. *Cancer Res* **56**: 892–898

Poikonen P, Saarto T, Lundin J, Joensuu H and Blomquist C (1999) Leucocyte nadir as a marker for chemotherapy efficacy in node positive breast cancer treated with adjuvant CMF. *Br J Cancer* (in press)

Saarto T, Blomquist C, Rissanen P, Auvinen A and Elomaa I (1997) Haematological toxicity: a marker of adjuvant chemotherapy efficacy in stage II and III breast cancer. *Br J Cancer* **75**: 301–305

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Prepubertal exposure to zearalenone or genistein reduces mammary tumorigenesis

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Summary Prepubertal exposure to a pharmacological dose (500 mg kg^{-1}) of the phyto-oestrogen genistein can reduce the incidence and multiplicity of carcinogen-induced mammary tumours in rats. However, such an exposure also disrupts the function of the hypothalamic–pituitary–gonadal axis, making it unsuitable for breast cancer prevention. We studied whether prepubertal exposure to genistein at a total body dose broadly comparable to the level typical of Oriental countries, approximately 1 mg kg^{-1} body weight, affects mammary tumorigenesis. We also studied whether prepubertal exposure to zearalenone, a major source for phyto-oestrogens in the USA, influences breast cancer risk. Prepubertal rats were treated between postnatal days 7 and 20, with 20 μg (~ 1 mg kg^{-1} body weight) of either genistein or zearalenone. Zearalenone exposure significantly reduced both the incidence and multiplicity of mammary tumours induced by 7,12-dimethylbenz(a)anthracene (DMBA). Genistein exposure significantly reduced tumour multiplicity, but not tumour incidence, when compared with vehicle-treated animals. Furthermore, 60% of the tumours in the genistein group were not malignant, while all the tumours analysed for histopathology in the vehicle and zearalenone groups were adenocarcinomas. A higher number of differentiated alveolar buds, and lower number of terminal ducts, were present in the DMBA-treated mammary glands of the phyto-oestrogen exposed rats. The concentration of oestrogen receptor (ER) binding sites after the DMBA treatment was low in the mammary glands of all groups but a significantly higher proportion of the glands in the zearalenone exposed rats were ER-positive (i.e. ER levels ≥ 5 fmol mg^{-1} protein) than the glands of the vehicle controls. Our data suggest that a prepubertal exposure to a low dose of either zearalenone or genistein may protect the mammary gland from carcinogen-induced malignant transformation, possibly by increasing differentiation of the mammary epithelial tree.

Keywords: genistein; zearalenone; prepuberty; mammary tumorigenesis

Asian populations with a high intake of phyto-oestrogens have a relatively low incidence of breast cancer (Setchell et al, 1984). Therefore, it has been suggested that phyto-oestrogens may reduce breast cancer risk (Messina et al, 1994). Phyto-oestrogens are naturally occurring compounds produced by a variety of plants. They are present in several foods, including soybean-based products that are typical for diets of Asia, but not for Western countries. Approximately 50% of the isoflavones in soybeans consists of genistein (Messina et al, 1994). There is some epidemiological evidence in favour of soy/genistein being anti-tumorigenic, but this is controversial. In three of a total of eight studies, a statistically significant association between high soy intake and low breast cancer risk has been found (Nomura et al, 1978; Hirohata et al, 1985; Lee et al, 1991; Yuan et al, 1995; Wu et al, 1996; Witte et al, 1997; Zheng et al, 1999). While most animal studies are supportive of the hypothesis that genistein inhibits mammary tumour promotion (Hawrylewicz et al, 1991; Messina et al, 1994; Barnes, 1997; Gotoh et al, 1998), some studies show an ability of genistein to increase mammary tumorigenesis (Hsieh et al, 1998). Prepubertal exposure via injections to a pharmacological dose of genistein (500 mg kg^{-1}) on days 16, 18 and 20 is reported to dramatically reduce subsequent risk to develop mammary tumours (Murrill et al, 1996). Thus, genistein may be particularly effective in reducing breast cancer risk, if its exposure occurs prior to puberty.

Zearalenone is another phyto-oestrogen that may be linked to mammary tumorigenesis. Zearalenone is mainly produced by the mould *Fusarium graminearum* found in a variety of host plants and debris from soil around the world (Burgess et al, 1982). It is present as a contaminant in stored cereals, being found in barley, corn, corn flakes, rice and wheat at concentrations from 35 to 115 μg kg^{-1} (Hagler et al, 1984; Schoental, 1985; Luo et al, 1990). Zearalenone also is used as an anabolic agent to enhance growth in cattle and lambs (Ralston, 1978; Wiggins et al, 1979). In contrast to genistein, pharmacological doses (10 mg kg^{-1} body weight) of zearalenone have been associated with an increased breast cancer risk in rats (Schoental, 1974).

Enthusiasm concerning the effects of prepubertal genistein exposure is limited because the dose used in previous studies is 5000 times higher than that of human exposure on a mg kg^{-1} body weight basis (Murrill et al, 1996). This pharmacological exposure causes severe perturbations in hypothalamic-ovarian function (Murrill et al, 1996) that may lead to infertility. Asians consume 2–9 g soy protein daily, while most women in Western countries do not consume any soy (Seow et al, 1998; Wu et al, 1998).

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Genistein content of different soy products varies considerably; for example, soy beans, soy milk and tofu contain approximately 2–18 μg genistein per g product, while miso and natto contain 40–330 μg genistein per g product (Lu et al, 1995; Fukutake et al, 1996). Fukutake et al (1996) have estimated that the daily genistein intake is 1.5–4.1 mg (< 0.1 mg kg^{-1}) among Asians. Zearalenone is the main phyto-oestrogen consumed in the USA (Kuiper-Goodman, 1990). The present study examined whether a prepubertal exposure to a more physiological dose of genistein and zearalenone (~ 1 mg kg^{-1} body weight) alters carcinogen-induced mammary tumorigenesis in rats. In addition, we investigated whether the concentrations of total oestrogen receptor (ER) binding sites are altered by prepubertal phyto-oestrogen administration. Low doses of genistein and zearalenone are known to bind to ER and affect its transcriptional regulatory activities (Wang et al, 1996; Collins et al, 1997; Zava and Duwe, 1997). The effect on mammary gland morphology also was studied, since a previous report suggested that an increased differentiation of the mammary epithelial tree by prepubertal genistein exposure may explain its cancer-reducing effects (Murrill et al, 1996).

METHODS

Animals

Pregnant female Sprague-Dawley rats, purchased from Charles Rivers Breeding Laboratories, were obtained at day 10 of gestation. The animals were housed singly, in standard rat plexiglas cages, at a constant temperature (20–22°C) and humidity (60–65%), under a 12-h light–dark cycle (lights on 06:00 h). Two days after the offspring were born, the males were sacrificed and the females cross-fostered. Ten to twelve female pups were housed with a lactating dam. The female offspring were weaned on postnatal day 22, and thereafter housed in groups of 3–5 animals. All studies were performed in accordance with the appropriate institutional and federal requirements.

Phyto-oestrogen exposure

On postnatal day 7, the litters were divided into three groups. The offspring received 20 μg genistein, 20 μg zearalenone (both from Sigma Chemical Co., St Louis, MO, USA), or vehicle, administered as subcutaneous injections, in a volume of 0.05 ml. Phyto-oestrogens were first dissolved in 2% dimethyl sulphoxide (DMSO), and then mixed with peanut oil (this mixture also served as vehicle). The injections were repeated on days 10, 14, 17 and 20. Body weight of a rat pup on day 7 is approximately 10 g and on day 20 25–30 g. Thus, the animals received genistein at the doses ranging from 2 mg kg^{-1} (day 7) to 0.7 mg kg^{-1} (day 20).

Inducing and monitoring of mammary tumorigenesis

Mammary tumors were induced by administration of 10 mg (~ 50 mg kg^{-1} body weight) 7,12-dimethylbenz(a)anthracene (DMBA) (Sigma, St Louis, MO, USA). This is a suboptimal dose used in our laboratory to enable assessments of both reductions and increases in the end points of tumorigenicity (Hilakivi-Clarke et al, 1997*b*). More than 75% of the tumours induced by 10 mg DMBA are adenocarcinomas (Russo and Russo, 1987). The carcinogen was dissolved in peanut oil and administered by oral gavage in a volume of 1 ml. Animals were 45 days old at the time of DMBA administration. The groups that were given phyto-oestrogens or vehicle after birth each contained 30 animals.

The animals were examined regularly for mammary tumours by palpation once per week. The end points for data analysis were (i) latency to tumour appearance, (ii) the number of animals with tumours (tumour incidence), (iii) the number of tumours per animal (tumour multiplicity), and (iv) tumour proliferation. A tumour was designated as proliferating if it increased regularly in size. Tumour sizes were measured by recording the tumour diameters with a caliper and determining the length of the longest axis and the width perpendicular to the longest axis. The animals were sacrificed when detectable tumour burden approximated 10% of total body weight, as required by our institution. All surviving animals, including those that did not appear to develop mammary tumours, were sacrificed 19 weeks after carcinogen administration.

Histopathology of the DMBA-induced mammary tumours was evaluated from 22 haematoxylin and eosin stained samples. Two pathologists at the Lombardi Cancer Center (Georgetown University) independently assessed the tumour samples. The pathologists were blind to the experimental groups.

Oestrogen receptor

The number of ER binding sites in the 4th mammary glands were determined from female rats exposed to genistein, zearalenone, or vehicle during the prepubertal period (n = 7 per group). The animals from which the mammary glands were taken had been treated with DMBA 18 weeks before, and consequently developed at least one mammary tumour. None of the tumours were in the 4th gland in the animals used for ER assays. ERs were detected using a ligand binding assay as described by Nelson et al (1986). The ligand used was [2,4,6,7-^{3}H] 17β oestradiol (specific activity 99 Ci $mmol^{-1}$; Amersham, Arlington Heights, IL, USA). This assay detects both ERα and ERβ with equal efficacy. Thus, ER binding reflects total ER concentrations (ERα + ERβ).

Mammary gland morphology

Whole mounts of the 9th mammary glands of the same female rats whose 4th glands were used for ER assays, were prepared (n = 4–5 per group). At the time of sacrifice, 18 weeks had passed from the DMBA administration. The removed glands were stained with carmine aluminium as previously described by Haslam (1988). We have previously validated a visual scale to study the development of a mouse and rat mammary epithelial tree (Hilakivi-Clarke et al, 1997*a*, 1997*b*). Using this scale, we determined differentiation of mammary epithelial structures in the whole mounts. The mammary epithelial trees were analysed for the density of ductal structures, terminal ducts and differentiated alveolar buds. This analysis was done double-blind under an Olympus dissecting microscope, using a 5-point scale (from 0 = absent to 5 = numerous). Differentiated alveolar buds do not give rise to adenocarcinomas. While terminal ducts occasionally give rise to tumours, the majority of tumours originate from terminal end buds (Russo and Russo, 1987). However, at the time the whole mounts were obtained from 6-month-old rats, all terminal end buds had differentiated to alveolar buds or regressed to terminal ducts. In addition to this quantitative analysis of the mammary whole mounts, a qualitative evaluation was performed.

Table 1 Effects of early postnatal exposure to 20 µg genistein or 20 µg zearalenone on mammary tumour growth

	Tumour latency (weeks)	Tumour area (mm^2)	Tumour multiplicity	Number of non-proliferating tumours
Vehicle (n = 33/17)[d]	11.0 ± 0.7	64.5 ± 13.5	1.8 ± 0.3	2 (6%)
Genistein (n = 15/13)	11.4 ± 0.3	67.7 ± 14.2	1.1 ± 0.1[b]	6 (40%)[c]
Zearalenone (n = 13/9)	14.4 ± 0.3[b]	58.7 ± 15.7	1.2 ± 0.1[a]	5 (38%)[c]

Significantly different from vehicle group: [a]$P < 0.06$, [b]$P < 0.01$, [c]$P < 0.001$. [d]n = Number of tumours/number of animals with tumours. Note: 60% of the tumours in the genistein group are benign, while all tumours examined in the vehicle and zearalenone groups are malignant adenocarcinomas. Data represent the mean ± s.e.m. of latency to tumour appearance, area of tumours at first detection, tumour multiplicity and percentage of non-proliferating tumours. Number of rats per group = 30.

Statistical analyses

Results for the data obtained on weight gain, ER binding sites, density of epithelial ducts, terminal ducts and alveolar buds in the whole mounts, and tumour latency, multiplicity, size upon first detection and growth data were analysed using one-way analysis of variance (Snedecor, 1988; Hanfelt, 1997). Where appropriate, between-group comparisons were done using Fisher's least significant difference. Results of tumour incidence were analysed using a log-rank survival analysis test. Differences in the number of non-proliferating and proliferating tumours, and the percentage of mammary glands that contained ER levels that were either ≥ 5 fmol mg^{-1} protein or < 5 fmol mg^{-1} protein, were determined using a χ^2 test. All probabilities are two-tailed. Statistical tests were performed using the BMDP software (BMDP Statistical Software, Los Angeles, CA, USA).

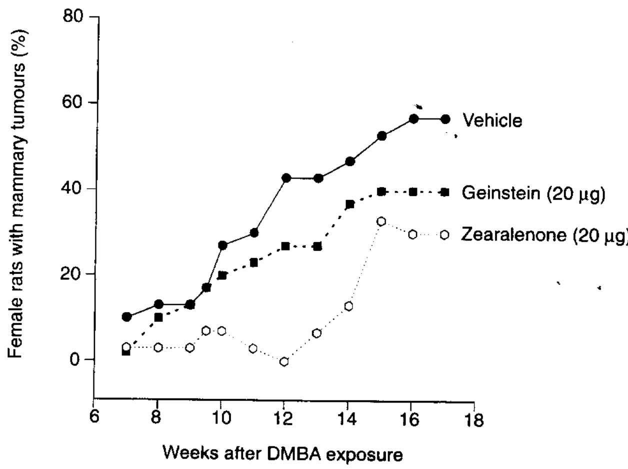

Figure 1 The proportion of female rats exposed to 20 µg genistein, 20 µg zearalenone, or vehicle during postnatal days 7, 10, 14, 17 and 20, that developed DMBA-induced (administered on day 45) mammary tumours. The number of animals per group was 30. Tumour incidence was significantly lower in the zearalenone-treated rats ($P < 0.001$)

RESULTS

Effect on weight gain

Early postnatal exposure to either genistein or zearalenone did not affect body weight gain. Between the first (day 7) and last day of phyto-oestrogen exposure (day 21), weight increased by 2.5-fold in the vehicle-treated rats, by 2.5-fold in the genistein-treated rats and by 2.6-fold in the zearalenone-treated rats. Body weights also were similar at the time the carcinogen was administered or 18 weeks after the administration (data not shown).

Mammary tumorigenesis

Tumour latency

The first tumours appeared on week 7 after the DMBA exposure in all groups. The mean tumour latency time to the first tumour per animal was significantly longer in the female rats exposed to zearalenone during early postnatal period than in the vehicle-treated rats ($F(2,37) = 4.91$, $P < 0.01$) (Table 1). Tumour latency was similar in the rats exposed to genistein or vehicle during prepuberty.

Tumour incidence

The incidence of mammary tumours (number of animals with tumours per group) was determined weekly, beginning on week 7 following DMBA administration. At the end of the study, on week 18 following DMBA administration, the percentage of rats with mammary tumours was 57% (17/30) in the vehicle-treated group, 43% (13/30) in the genistein-treated group, and 30% (9/30) in the zearalenone-treated group ($\chi^2 = 14.92$, df = 2, $P < 0.001$). Log-rank test analysis indicated that the tumour incidence was significantly lower in the animals exposed to zearalenone during the prepubertal period than in the vehicle-treated controls (z-value = 2.36, $P < 0.018$). The slightly lower tumour incidence in the genistein-exposed rats was not statistically significant, when compared with the controls ($z = 1.03$, $P < 0.30$) (Figure 1).

Tumour multiplicity

The average number of tumours per animal was significantly lower in the rats exposed to genistein ($P < 0.01$) during prepubertal life than in the vehicle-treated rats ($F(2,38) = 4.53$, $P < 0.02$). A reduction that approached statistical significance also was seen in the zearalenone-exposed rats ($P < 0.06$) (Table 1).

Tumour growth rate

The size of the tumours upon first detection was similar among the groups (Table 1). However, the percentage of proliferating tumours in the genistein- (60%) and zearalenone-exposed rats (62%) was significantly lower than in the vehicle-treated group (94%) ($\chi^2 = 9.96$, df = 2, $P < 0.001$).

Tumour histopathology

Histopathological analysis performed for 22 samples indicated that the histotypes of all mammary tumours in the vehicle- and zearalenone-treated groups were adenocarcinomas (100%). In the

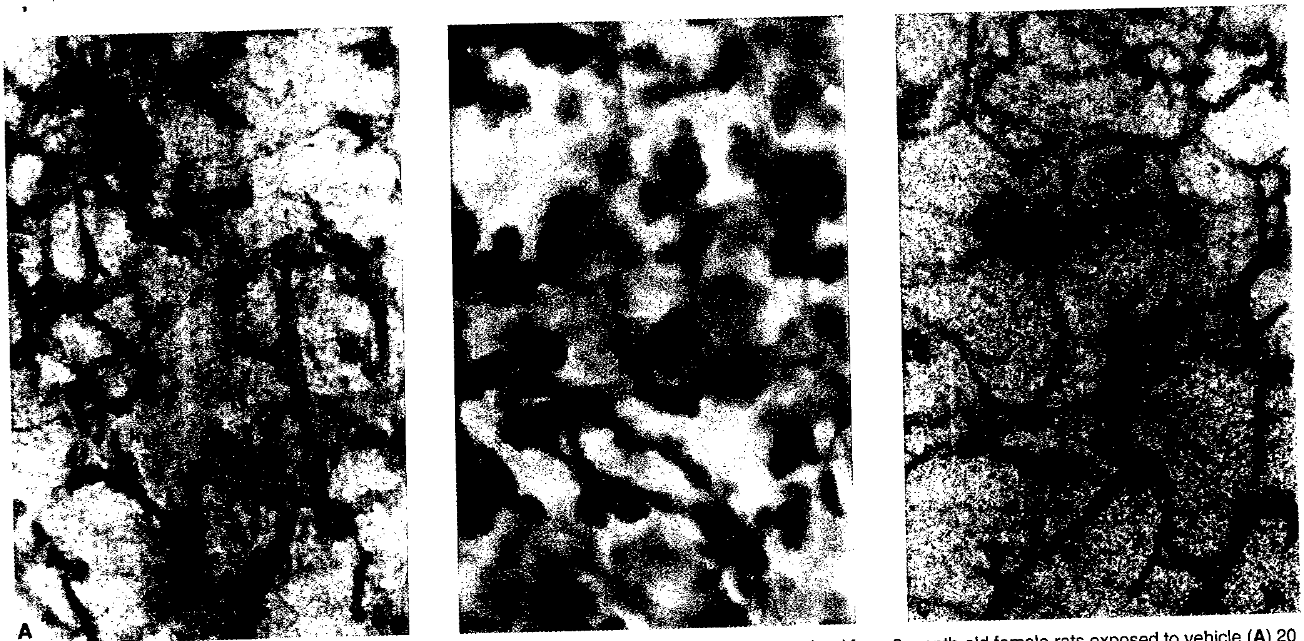

Figure 2 Mammary wholemount preparations (carmine staining) of the 9th abdominal glands obtained from 6-month-old female rats exposed to vehicle (**A**) 20 μg genistein (**B**) or 20 μg zearalenone (**C**) during a prepubertal period, and to DMBA at the age of 45 days. This figure is representative of 4–5 specimens per group. The wholemounts of the genistein group contained high levels of 2–3 type lobules, and the whole mounts of zearalenone group indicated ductal atrophy, combined with higher level of lobular structures than seen in the vehicle group (but clearly less than in the genistein group). Magnification x 6.3

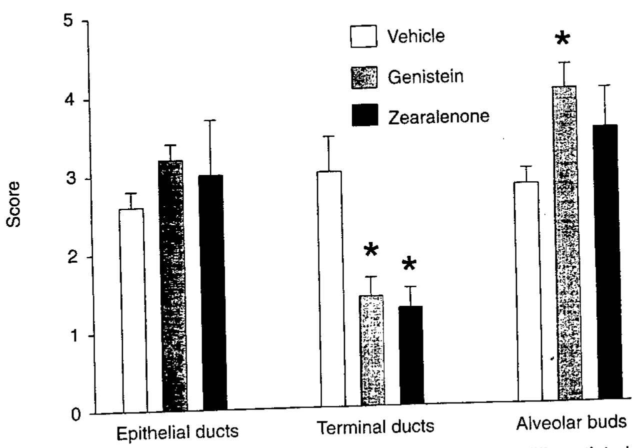

Figure 3 Density of epithelial ducts, terminal ducts (TDs) and differentiated alveolar buds (ABs) of the 9th abdominal gland obtained from 6-month-old female rats exposed to 20 μg genistein, 20 μg zearalenone, or vehicle during a prepubertal period, and to DMBA at the age of 45 days (n = 4–5 female rats per group). These parameters were evaluated using a scoring system with scores ranging from 0 (absent) to 5 (high), as described in Methods. Means ± s.e.m. are shown. Significantly different from the vehicle group: $^*P < 0.05$

genistein-treated group, 40% of the tumours were adenocarcinomas and 60% were non-malignant.

Oestrogen receptor

The concentrations of ER protein were determined in the mammary glands in rats that were exposed to DMBA 18 weeks prior to sacrifice. The ER levels were similar in the animals treated with genistein (3.4 ± 1.4 fmol mg^{-1} protein) or zearalenone (4.3 ± 2.8 fmol mg^{-1} protein) during the prepubertal period, and did not differ from that of the controls (3.9 ± 1.7 fmol mg^{-1} protein). The total ER levels in the present study were lower than we have seen in mice (Hilakivi-Clarke et al, 1998), but comparable to those seen in rats exposed to carcinogens (Thordarson et al, 1995).

We also determined the percentage of mammary glands that had ER levels higher than 5 fmol mg^{-1}. Breast tumours containing ER levels ≥ 5 fmol mg^{-1} protein are often considered ER-positive (Clark and McGuire, 1988; Winstanley et al, 1991). Using this cut-off point the data indicate that the proportion of ER-positive glands was significantly higher in the zearalenone-treated group (43%) than in the genistein- (14%) or vehicle-treated (28%) groups (χ^2 = 17.80, df = 2, $P < 0.01$).

Mammary whole mounts

Analysis of mammary whole mounts obtained from animals that had been exposed to DMBA 18 weeks prior to sacrifice, indicated that prepubertal exposure to genistein and zearalenone induced distinct differences, when compared with the vehicle-controls (Figure 2). The mammary glands of genistein-treated animals showed the most lobular differentiation. These lobules were of type 2 and 3, indicating a high level of differentiation. Zearalenone-treated glands also showed differentiated lobular structures, although less than that seen in the genistein-treated glands.

According to the scale-based quantitative evaluation, total mammary epithelial density did not differ among the groups (Figure 3). However, the density of terminal ducts was significantly lower in the rats exposed to zearalenone ($P < 0.05$) or genistein ($P < 0.05$) during the prepubertal period than in the vehicle controls ($F(2,11) = 8.30$, $P < 0.006$). The density of alveolar buds, in turn, was significantly higher in the genistein-treated group ($P < 0.05$) than in the controls ($F(2,11) = 3.44$, $P < 0.07$).

DISCUSSION

Despite the general perception that consumption of soy-based food products protects from breast cancer, the supporting epidemiological evidence is inconsistent. Three studies suggest that soy intake is associated with lower breast cancer risk. A study in Singaporean women found that high soy intake is associated with a lower breast cancer risk among premenopausal, but not postmenopausal, women (Lee et al, 1991). A study in Asian-American women living in Los Angeles or Hawaii indicated that breast cancer risk decreases with increasing frequency of intake of tofu (bean curd) both in pre- and postmenopausal women (Wu et al, 1996). Finally, a study that measured urinary excretion levels of phyto-oestrogens reported that a high excretion of isoflavones (genistein was not included) was associated with a substantial reduction in breast cancer risk (Ingram et al, 1997). A similar, but more recent, study did not find significant differences in urinary excretion levels of daidzein or genistein between breast cancer cases and their controls in Shanghai; however, total isoflavonoid levels were lower in the cases (Zheng et al, 1999). Soy protein intake was similar in these Shanghai women who were newly diagnosed with breast cancer and randomly selected controls. Four other studies also suggest that the risk of breast cancer is not associated with soy consumption (Hirohata et al, 1985; Yuan et al, 1995; Witte et al, 1997). These significant inconsistencies may reflect differences in the end points used for genistein intake (consumption of tofu, miso, or serum isoflavone concentrations). It also is possible that timing of genistein exposure might be critical.

Treatment during prepuberty, with a pharmacological dose of genistein, has been suggested to reduce the subsequent risk to develop breast cancer. Murrill et al (1996) found that a subcutaneous exposure of prepubertal rats on postnatal days 16, 18 and 20 to 500 mg kg^{-1} genistein significantly reduces mammary tumour incidence and multiplicity. However, this dose is 500–5000 times higher than human genistein intake, and may not be directly relevant to human populations. We have used a genistein dose of ~1 mg kg^{-1} body weight, which should approximate the daily genistein consumption in Asia on a mg kg^{-1} body weight basis. If this dose is further adjusted for interspecies surface area differences (Freireich et al, 1966; Clarke, 1997), a human genistein exposure equivalent to 0.143 mg kg^{-1} body weight is obtained, comparable to the exposure to genistein alone in Oriental populations (~ 0.1 mg kg^{-1} body weight). Our data show that when the rats were exposed to this substantially lower genistein dose between postnatal days 7 and 20, they exhibited reduced mammary tumour multiplicity, but no significant change in tumour incidence. Additionally, a significant proportion of the tumours in the genistein group did not proliferate, and 60% of them were not malignant. Thus, prepubertal exposure to genistein not only reduced the subsequent mammary tumour multiplicity, it also reduced the likelihood that a tumour was malignant.

The lack of significance in the tumour incidence and latency in the genistein-exposed rats in the present study, when compared with the previous study (Murrill et al, 1996), is likely to be caused by the use of a low versus high dose of genistein. Genistein displays a convex dose–response curve for oestrogenic activity. Low genistein doses stimulate ER, while higher doses inhibit the receptor's activity (Wang et al, 1996). Inhibition of ER by high genistein doses may be due to inhibition of the tyrosine kinase activity of the epidermal growth factor receptor (EGFR) (Akiyama et al, 1987), which could lead to a reduced phosphorylation of ER. Another means by which pharmacological doses of genistein may affect tumorigenesis is by influencing the reproductive system. The 500 mg kg^{-1} genistein administration to prepubertal rats caused a permanent impairment of the hypothalamic–gonadal axis (Murrill et al, 1996). It also has been reported that 1 mg genistein (~ 100 mg kg^{-1}) given to rat pups daily between postnatal days 1 and 10, significantly decreases pituitary responsiveness to gonadotrophin releasing hormone (GnRH) (Faber and Hughes, 1991). A ten times lower genistein dose (100 μg ≈ 10 mg kg^{-1}) has the opposite effect, increasing GnRH-induced secretion of luteinizing hormone (Faber and Hughes, 1991). Thus, changes in the magnitude of a genistein exposure may have opposing effects on the reproductive system. We did not perform a detailed analysis of the reproductive system functions, and therefore cannot exclude the possibility that a low genistein dose (1 mg kg^{-1}) disrupts reproductive functions. However, we did not observe any differences in the timing of the onset of sexual maturation with prepubertal exposure to either genistein or zearalenone (not shown), which would be one measure of altered reproductive function.

Zearalenone, when administered during the prepubertal period, effectively reduced DMBA-induced mammary tumorigenesis in female rats. Rats that received 20 μg (~ 1 mg kg^{-1}) zearalenone every 3–4 days between days 7 and 20, subsequently exhibited a longer latency to tumour appearance, and had a lower tumour incidence than the vehicle-treated controls. In addition, one-third of the tumours did not proliferate. However, histopathological evaluation indicated that all the assessed tumours in the zearalenone group were malignant. These findings contrast with earlier data obtained in rats showing that treatment on postnatal days 7 and 14 with 10 mg kg^{-1} zearalenone increases the incidence of spontaneous mammary tumours (Schoental, 1985). The opposite results may be caused by the tenfold difference in the dose of zearalenone used in the two studies. We also treated the rats with a carcinogen and measured tumorigenesis within the following 18 weeks, while in the other study spontaneously arising tumours appeared when the animals were 2 years of age. These differences also may indicate that prepubertal zearalenone exposure inhibits premenopausal breast cancer (that is mimicked by DMBA), and stimulates the growth of postmenopausal breast cancer (that is mimicked by spontaneous tumours in older rats).

Both genistein and zearalenone act as relatively weak oestrogens. They stimulate the growth of human breast cancer cells in vitro (Martin et al, 1978; Wang et al, 1996), and have similar properties to oestradiol in rodent and human breast tissues (Petrakis et al, 1996; Harding et al, 1997; Santell et al, 1997; Hsieh et al, 1998; McMichael-Phillips et al, 1998). Since oestrogens are thought to increase breast cancer risk (Clarke et al, 1992), it is surprising that genistein or zearalenone in the present and previous study (Murrill et al, 1996) reduced the incidence/multiplicity of mammary tumours. Perhaps oestrogens have opposing effects on breast cancer risk depending on the timing of exposure. In utero exposure to oestrogens, or an exposure during the first week after birth, increases the subsequent incidence of mammary tumours in mice and rats (Bern et al, 1985; Lopez et al, 1988; Hilakivi-Clarke et al, 1997*b*), and possibly also in humans (Ekbom et al, 1992; Michels et al, 1996; Sanderson et al, 1996). However, prepubertal or pubertal treatment with oestradiol reduces mammary tumorigenesis in rats (Nagasawa et al, 1974; Grubbs et al, 1985). An exposure to oestrogens during adulthood increases breast cancer risk in animals (Clarke et al, 1992; Russo et al, 1994), and perhaps in humans (Grodstein et al, 1997). These differences may reflect

changes in the responsiveness of the mammary gland to ER oestrogens over time.

In the present study, phyto-oestrogen exposure occurred during a prepubertal period when the mammary epithelial cells are thought not to respond fully to oestrogens, e.g. oestrogens do not cause epithelial cell proliferation or alter ER levels (Haslam, 1989). We did not find any evidence that the prepubertal exposure to genistein or zearalenone permanently affected the levels of ER in the mammary gland. However, the mammary glands of the zearalenone-treated rats were significantly more often ER-positive (had ER levels $\geq$ 5 fmol mg^{-1} protein) than those of the genistein group. Our findings are consistent with the fact that ligands can alter the number/function of a receptor when they are present, and generally do not cause any persistent changes in receptor expression. One exception is an exposure that occurs during fetal life or immediately after birth, which can produce a permanent change (Verhoeven et al, 1982; Bern et al, 1985; Hilakivi-Clarke et al, 1998). However, since a prepubertal treatment with both a low (interacts with ER) and high (interacts with ER and other targets) dose of genistein or zearalenone reduces subsequent mammary tumorigenesis, prepubertal ERs may be involved as mediators of these effects.

Whether or not the effects we report are a direct consequence of changes in oestrogen-regulated gene function, it seems highly likely that the mechanism for prepubertal oestrogen exposure in affecting breast cancer risk involves changes in the mammary epithelial network. We have proposed that the increased number of terminal end buds and their reduced differentiation to alveolar buds, plays a critical role in increasing breast cancer risk following a perinatal oestrogen exposure (Hilakivi-Clarke et al, 1997*a*). Terminal end buds, and to a lesser degree terminal buds, are the primary sites of carcinogen action in the mammary gland (Russo and Russo, 1987). Consistent with this hypothesis, prepubertal exposure to a pharmacological genistein dose increases differentiation of terminal end buds at the time when DMBA is administered (Murrill et al, 1996). In our study, a more physiological dose of genistein also increased differentiated alveolar buds, when determined 18 weeks after DMBA exposure.

In summary, prepubertal exposure to a low dose of genistein or zearalenone reduces the risk to develop mammary tumours in rats. The mediating mechanisms remain to be established, but are likely to include changes in the differentiation of mammary epithelial tree and may reflect events mediated through activation of the ER.

REFERENCES

Akiyama T, Ishida J, Nakagawa S, Ogawa H, Watanabe S, Itou N, Shibata M and Fukami Y (1987) Genistein, a specific inhibitor of tyrosine-specific protein kinase. *J Biol Chem* **262**: 5592–5595

Barnes S (1997) The chemopreventive properties of soy isoflavonoids in animal models of breast cancer. *Breast Cancer Res Treat* **46**: 169–179

Bern HA, Mills KT and Edery M (1985) Estrogen-associated defects in rodent mammary gland development. In: *Estrogens in the Environment*, McLachlan JA (ed), pp. 319–326 Elsevier: Amsterdam

Burgess LW, Nelson PE and Toussoun TA (1982) Characterization, geographic distribution and ecology of Fusarium crookwellense sp. nov. *Trans Br Mycol Soc* 79: 497–50[illegible]

Clark GM and McGuire WL (1988) Steroid receptors and other prognostic factors in primary breast cancer. *Semin Oncol* **15**: 20–25

Clarke R (1997) Issues in experimental design and analysis in the study of experimental cytotoxic agents in vivo in breast cancer and other models. *Breast Cancer Res Treat* **46**: 255–278

Clarke R, Dickson RB and Lippman ME (1992) Hormonal aspects of breast cancer. Growth factors, drugs and stromal interactions. *Crit Rev Oncol Hematol* **12**: 1–23

Collins BM, McLachlan JA and Arnold SF (1997) The estrogenic and antiestrogeneic activities of phytochemicals with the human estrogen receptor expressed in yeast. *Steroids* **62**: 365–372

Ekbom A, Trichopoulos D, Adami HO, Hsieh CC and Lan SJ (1992) Evidence of prenatal influences on breast cancer risk. *Lancet* **340**: 1015–1018

Faber KA and Hughes CLJ (1991) The effect of neonatal exposure to diethylstilbestrol, genistein, and zearalenone on pituitary responsiveness and sexually dimorphic nucleus volume in the castrated adult rat. *Biol Reprod* **45**: 649–653

Freireich EJ, Gehan EA, Rall DP, Schmidt LH and Skipper HE (1966) Quantitative comparison of toxicity of anticancer agents in the mouse, rat, hamster, dog, monkey and man. *Cancer Chemother Rep* **50**: 219–244

Fukutake M, Takahashi M, Ishida K, Kawamura H, Sugimura T and Wakabayashi K (1996) Quantification of genistein and genistin in soybeans and soybean products. *Food Chem Toxicol* **34**: 457–461

Gotoh T, Yamada K, Yin H, Ito A, Kataoka T and Dohi K (1998) Chemoprevention of N-nitroso-N-methylurea-induced rat mammary carcinogenesis by soy foods or biochanin A. *Jpn J Cancer Res* **89**: 137–142

Grodstein F, Stampfer MJ, Colditz GA, Willett WC, Manson JE, Joffe M, Rosner B, Fuchs C, Hankinson SE, Hunter DJ, Hennekens CH and Speizer FE (1997) Postmenopausal hormone therapy and mortality. *N Eng J Med* **336**: 1769–1775

Grubbs CJ, Farneli DR, Hill DL and McDonough KC (1985) Chemoprevention of n-nitro-n-methylurea-induced mammary cancers by pretreatment with 17beta-estradiol and progesterone. *J Natl Cancer Inst* **74**: 927–931

Hagler WM, Tyczkowska K and Hamilton PB (1984) Simultaneous occurrence of deoxynivalenol, zearalenone, and aflatoxin in 1982 scabby wheat from the Midwestern United States. *Appl Environ Microbiol* **47**: 151–154

Hanfelt JJ (1997) Statistical approaches to experimental design and data analysis of in vivo studies. *Breast Cancer Res Treat* **46**: 279–302

Harding C, Tetlow L, McMichael Phillips D, Osundeko O, Potten CS and Bundred NJ (1997) Oestrogenic effects of soy on nipple aspirate fluid. *Breast Cancer Res Treat* **46**: 80-Abstract

Haslam SZ (1988) Progesterone effects on deoxyribonucleic acid synthesis in normal mouse mammary glands. *Endocrinology* **122**: 464–470

Haslam S (1989) The ontogeny of mouse mammary gland responsiveness to ovarian steroid hormones. *Endocrinology* **125**: 2766–2772

Hawrylewicz EJ, Huang HH and Blair WH (1991) Dietary soybean isolate and methionine supplementation affect mammary tumor progression in rats. *J Nutr* **121**: 1693–1698

Hilakivi-Clarke L, Cho E, Raygada M and Kenney N (1997*a*) Alterations in mammary gland development following neonatal exposure to estradiol, transforming growth factor alpha, and estrogen receptor antagonist ICI 182, 780. *J Cell Physiol* **170**: 279–289

Hilakivi-Clarke L, Clarke R, Onojafe I, Raygada M, Cho E and Lippman ME (1997*b*) A maternal diet high in n-6 polyunsaturated fats alters mammary gland development, puberty onset, and breast cancer risk among female rat offspring. *Proc Natl Acad Sci USA* **94**: 9372–9377

Hilakivi-Clarke LA, Raygada M, Stoica A and Martin M-B (1998) Consumption of a high-fat diet during pregnancy alters estrogen receptor content, protein kinase C activity and morphology of mammary gland in the mother and her female offspring. *Cancer Res* **58**: 654–660

Hirohata T, Shigematsu T, Nomura AMY, Nomura Y, Horie A and Hirohata I (1985) Occurrence of breast cancer in relation to diet and reproductive history: a case-control study in Fukuoka, Japan. *Natl Cancer Inst Monogr* **69**: 187–190

Hsieh CY, Santell RC, Haslam SZ and Helferich WG (1998) Estrogenic effects of genistein on the growth of estrogen receptor-positive human breast cancer (MCF-7) cells in vitro and in vivo. *Cancer Res* **58**: 3833–3838

Ingram D, Sanders K, Kolybaba M and Lopez D (1997) Case-control study of phytoestrogens and breast cancer. *Lancet* **350**: 990–994

Kuiper-Goodman T (1990) Uncertainties in the risk assessment of three mycotoxins: aflatoxin, ochratoxin, and zearalenone. *Can J Physiol Pharm* **68**: 1017–1024

Lee HP, Gourley L, Duffy SW, Esteve J, Lee J and Day NE (1991) Dietary effects on breast cancer risk in Singapore. *Lancet* **337**: 1197–1200

Lopez J, Ogren L, Verjan K and Talamantes F (1988) Effects of perinatal exposure to a synthetic estrogen and progestin on mammary tumorigenesis in mice. *Teratology* **38**: 129–134

Lu LJ, Broemeling L, Marshall M and Ramanujam VM (1995) A simplified method to quantify isoflavones in commercial soybean diets and human urine after legume consumption. *Cancer Epidemiol Biomarkers Prev* **4**: 497–503

Luo Y, Yoshizawa T and Katayama T (1990) Comparative study on the natural occurrence of fusarium mycotoxins (trichothecenes and zearalenone) in corn and wheat from high- and low-risk areas for human esophageal cancer in China. *Appl Environ Microbiol* **56**: 3723–3726

McMichael-Phillips DF, Harding C, Morton M, Roberts SA, Howell A, Potten CS and Bundred NJ (1998) Effect of soy-protein supplementation on epithelial proliferation in the histologically normal human breast. *Am J Clin Nutr* **68** (suppl), 1431S–1436S

Martin PM, Horwitz KB, Ryan DS and McGuire W (1978) Phytoestrogen interaction with estrogen receptors in human breast cancer cells. *Endocrinology* **103**: 1860–1867

Messina M, Persky V, Setchell KDR and Barnes S (1994) Soy intake and breast cancer: a review of the in vitro and in vivo data. *Nutr Cancer* **21**: 113–131

Michels KB, Trichopoulos D, Robins JM, Rosner BA, Manson JE, Hunter D, Colditz GA, Hankinson SE, Speizer FE and Willett WC (1996) Birth weight as a risk factor for breast cancer. *Lancet* **348**: 1542–1546

Murrill WB, Brown NM, Zhang JX, Manzolillo PA, Barnes S and Lamartiniere CA (1996) Prepubertal genistein exposure suppresses mammary cancer and enhances gland differentiation in rats. *Carcinogenesis* **17**: 1451–1457

Nagasawa H, Yanai R, Shonodo M, Nakamura T and Tanabe Y (1974) Effect of neonatally administered estrogen and prolactin on normal and neoplastic mammary growth and serum estradiol-17 level in rats. *Cancer Res* **34**: 2643–2646

Nelson J, Clarke R, Dickson GR, Van Den Berg HW and Murphy RF (1986) The effects of Mg2+ ions or EDTA on nuclear integrity and apparent subcellular distribution of unoccupied oestrogen receptors in breast cancer cells. *J Steroid Biochem* **25**: 619–626

Nomura A, Henderson BE and Lee J (1978) Breast cancer and diet among the Japanese in Hawaii. *Am J Clin Nutr* **31**: 2020–2025

Petrakis NL, Barnes S, King EB, Lowenstein J, Wiencke J, Lee MM, Miike R, Kirk M and Coward L (1996) Stimulatory influence of soy protein isolate on breast secretion in pre- and postmenopausal women. *Cancer Epidemiol Biomark Prev* **5**: 785–794

Ralston AT (1978) Effect of zearalanol on weaning weight of male calves. *J Anim Sci* **47**: 1203–1206

Russo IH, Medado J and Russo J (1994) Endocrine influences on the mammary gland. In *Integument and Mammary Glands*. Eds TC Jones, U Mohr & RD Hunt. Berlin: Springer-Verlag. pp. 252–266

Russo J and Russo IH (1987) Biological and molecular bases of mammary carcinogenesis. *Lab Invest* **57**: 112–137

Sanderson M, Williams M, Malone KE, Stanford JL, Emanuel I, White E and Daling JR (1996) Perinatal factors and risk of breast cancer. *Epidemiology* **7**: 34–37

Santell RC, Chang YC, Nair MG and Helferich WG (1997) Dietary genistein exerts estrogeneic effects upon the uterus, mammary gland and the hypothalamic/pituitary axis in rats. *J Nutr* **127**: 263–269

Schoental R (1974) Role of podophyllotoxin in the bedding and dietary zearalenone on incidence of 'spontaneous' tumors in laboratory animals. *Cancer Res* **34**: 2419

Schoental R (1985) Trichothecenes, Zearalenone, and other carcinogenic metabolites of fusarium and related microfungi. *Adv Cancer Res* **45**: 217–290

Seow A, Shi C-Y, Franke A, Hankin JH, Lee HP and Yu MC (1998) Isoflavoid levels in spot urine are associated with frequency of dietary soy intake in a population-based sample of middle-aged and older Chinese in Singapore. *Cancer Epidemiol Biomarkers Prev* **7**: 135–140

Setchell KDR, Borriello SP, Hulme P, Kirk DN and Axelson M (1984) Nonsteroidal estrogens of dietary origin: possible roles in hormone-dependent disease. *Am J Clin Nutr* **40**: 569–578

Snedecor GW (1988) *Statistical Methods*, 8th Edn. Iowa State University Press: Ames, Iowa

Thordarson G, Jin E, Guzman RC, Swanson SM, Nandi S and Talamantes F (1995) Refractoriness to mammary tumorigenesis in parous rats: is it caused by persistent changes in the hormonal environment or permanent biochemical alterations in the mammary epithelia? *Carcinogenesis* **16**: 2847–2853

Verhoeven G, Vandoren G, Heyns W, Kuhn ER, Janssens JP, Teuwen D, Goddeeris E, Lesaffre E and DeMoor P (1982) Incidence, growth and estradiol-receptor levels of 7,12-dimethylbenz(alpha)antracene-induced mammary tumours in rats: effects of neonatal sex steroids and oestradiol implants. *J Endocrinol* **95**: 357–368

Wang TT, Sathyamoorthy N and Phang JM (1996) Molecular effects of genistein on estrogen receptor mediated pathways. *Carcinogenesis* **17**: 271–275

Wiggins JP, Rothenbacher H, Wilson LL, Martin RJ, Wangness PJ and Ziegler JH (1979) Growth and endocrine responses of lambs to zearanol implants: effects of preimplant growth rate and breed of sire. *J Anim Sci* **49**: 291–297

Winstanley J, Cooke T, George WD, Murray G, Holt S, Croton R, Griffiths K and Nicholson R (1991) The long term prognostic significance of oestrogen receptor analysis in early carcinoma of the breast. *Br J Cancer* **64**: 99–101

Witte JS, Ursin G, Siemiatycki J, Thompson WD, Paganini-Hill A and Haile RW (1997) Diet and premenopausal bilateral breast cancer: A case-control study. *Breast Cancer Res Treat* **42**: 243–251

Wu AH, Ziegler RG, Horn-Ross PL, Nomura AMY, West DW, Kolonel LN, Rosenthal JF, Hoover RN and Pike MC (1996) Tofu and risk of breast cancer in Asian-Americans. *Cancer Epidemiol Biomarkers Prev* **5**: 901–906

Wu AH, Ziegler RG, Nomura AMY, West DW, Kolonel LN, Horn-Ross PL, Hoover RN and Pike MC (1998) Soy intake and risk of breast cancer in Asians and Asian-Americans. *Am J Clin Nutr* **68** (Suppl), 1437S–1443S

Yuan JM, Wang QS, Ross RK, Henderson BE and Yu MC (1995) Diet and breast cancer in Shanghai and Tianjin, China. *Br J Cancer* **71**: 1353–1358

Zava DT and Duwe G (1997) Estrogenic and antiproliferative properties of genistein and other flavonoids in human breast cancer cells in vitro. *Nutr Cancer* **27**: 31–40

Zheng W, Dai Q, Cluster LJ, Shu XO, Wen WQ, Jin F and Franke AA (1999) Urinary excretion of isoflavonoids and the risk of breast cancer. *Cancer Epidemiol Biomarkers Prev* **8**: 35–40

Cellular and Molecular Pharmacology of Antiestrogen Action and Resistance

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Running Title Page

Running Title: *"Antiestrogen Action and Resistance"*

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Nonstandard abbreviations: Antiestrogen binding site, AEBS; cell mediated immunity, CMI; epidermal growth factor, EGF; estrogen receptor, ER; fibroblast growth factor, FGF; glucocorticoid receptor, GR; insulin-like growth factor, IGF; insulin-like growth factor binding protein, IGF-BP;lymphokine activated killer, LAK; mitogen activated kinase, MAPK; natural killer, NK; progesterone receptor, PgR; protein kinase C, PKC; selective estrogen receptor modulator, SERM; stress activated protein kinase, SAPK; Tamoxifen, TAM; transforming growth factor, TGF.

Abstract

Antiestrogen therapy remains one of the most widely used and effective treatments for the management of endocrine responsive breast cancers. This reflects the ability of antiestrogens to compete with estrogens for binding to estrogen receptors. While response rates of up to 70% are reported in patients with tumors expressing estrogen and progesterone receptors, most responsive tumors will eventually acquire resistance. The most important factor in *de novo* resistance is lack of expression of these receptors. However, the mechanisms driving resistance in tumors that express estrogen and/or progesterone receptors are unclear. A tamoxifen stimulated phenotype has been described but appears to occur only in a minority of patients. Most tumors (>80%) may become resistant through other, less well defined, resistance mechanisms. These may be multifactorial, including changes in immunity, host endocrinology and drug pharmacokinetics. Significant changes within the tumor cells also may occur, including alterations in the ratio of the estrogen receptor α:β forms, and/or other changes in estrogen receptor-driven transcription complex function. These may lead to perturbations in the gene network signaling downstream of estrogen receptors. Cells also may alter paracrine and autocrine growth factor interactions, potentially producing a ligand-independent activation of estrogen receptors by mitogen activated protein kinases. Antiestrogens can affect the function of intracellular proteins and signaling that may, or may not, involve estrogen receptor-mediated events. These include changes in oxidative stress responses, specific protein kinase C isoform activation, calmodulin function, and cell membrane structure/function.

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CHAPTER 23

In Vitro Models

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Breast cancer cells that grow *in vitro* represent one of the most widely used experimental models of breast cancer. For many studies, these models provide the only means to address a specific hypothesis. Most breast cancer cell lines can easily be maintained and studied *in vitro*, and are generally stable with respect to their endocrine responsiveness *in vitro* and *in vivo*. The current understanding of how breast cancer cells respond to estrogenic stimuli is, in no small part, the direct result of *in vitro* studies with human breast cancer cell lines.

Breast cancer cell lines are generally considered in terms of their estrogen-receptor (ER) content—that is, whether they are estrogen receptor–positive (ER+) or estrogen receptor–negative (ER–). This classification largely reflects the clinical value of steroid hormone expression in predicting response to endocrine therapy. However, other characteristics of human breast tumors that tend to follow ER status are frequently exhibited in a similar pattern by cell lines growing *in vitro* and *in vivo*.[1]

A detailed and inclusive review of all breast cancer cell lines and their origins, characteristics, and uses is beyond the scope of this chapter. Consequently, the chapter focuses primarily on the most widely used cell lines, some of their variants, and those models expressing characteristics that closely reflect many of the properties of breast tumors in patients. Also included is a brief description of cell lines that have more unique properties or that are best suited to specific studies, such as endocrine regulation or expression of growth factors or oncogenes. The parental cell lines are readily available (e.g., several are available through the American Type Culture Collection in Rockville, Maryland), and the variants can generally be obtained from their respective originators. For a more detailed description of the characteristics of several of these cell lines, the reader is referred to the review by Engel and Young.[2] Although more than two decades old, this review provides valuable information on the origin and characteristics of 22 breast cancer cell lines. The inclusion of the original citations for almost all of these cell lines provides a valuable reference for the interested reader.

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ESTROGEN-DEPENDENT (ER+/PR+) BREAST CANCER CELL LINES

Approximately 30% of all breast cancer patients respond to endocrine manipulation. The overall response rate to antiestrogens increases to 70% or more in patients whose tumors express both the estrogen receptor (ERs) and progesterone receptor (PR).[3–5] To define the mechanism of action of endocrine therapies and to develop and screen new agents and therapies require models that exhibit an endocrine response profile comparable to that found in breast cancer patients. In this regard, the steroid-dependent breast cancer cell lines have been most useful in studying the growth-inhibitory effects of estrogens, antiestrogens, progestins, and antiprogestins. These cell lines are characterized by a dependence on estrogens for growth *in vitro* or *in vivo* and by sensitivity to the growth-inhibitory effects of antiestrogenic and progestational drugs. In general, steroid-dependent cell lines are poorly invasive and nonmetastatic in athymic nude mice.

MCF-7

The MCF-7 cell line is the most widely used and best characterized of all the human breast cancer cell lines. The mitogenic effects of 17β-estradiol (E2) in human breast cancer cells *in vitro* were initially defined in these cells, as were the *in vitro* inhibitory effects of antiestrogens.[6,7] MCF-7 cells also are growth inhibited by luteinizing hormone–releasing hormone (LHRH) analogues[8] and retinoids.[9–11] The now widely reported E2 dependence for exponential growth both *in vitro* and *in vivo* has provided this cell line with a central role in the study of endocrine responsiveness and malignant progression *in vitro*. Much of the current understanding of the mechanism of action of estrogens and antiestrogens and their role in regulating the proliferation of hormone-dependent breast cancer cells has been derived from work performed using this cellular model. Consequently, a full and detailed discussion of all

the data generated using MCF-7 cells alone is beyond the scope of this chapter.

MCF-7 cells were established from a pleural effusion arising in a postmenopausal woman with breast cancer. The patient had received radiotherapy and endocrine therapy before the appearance of the effusion.[12] In addition to the expression of ER,[12,13] MCF-7 cells express an E2-inducible PR[13,14] and cellular receptors for androgens,[13] LHRH,[8,15] glucocorticoids,[13] insulin,[16] retinoic acid receptors (RAR-α and RAR-γ),[17] and prolactin.[18] The expression/secretion of several growth factors and their receptors also has been described in detail, including the insulinlike growth factors (IGFs),[19–22] type I and type II IGF receptors,[22–24] and several IGF binding proteins[25–27]; transforming growth factor α (TGF-α) and epidermal growth factor receptor (EGFR)[28–30]; several fibroblast growth factors (FGFs)[31] and FGF receptors[32]; and platelet-derived growth factor (PDGF) but not PDGF receptors.[33] The expression of many of these growth factors, and their respective receptors, is strongly E2 regulated in MCF-7 cells.[34] The expression, secretion, and regulation of this wide variety of receptors and ligands has made the MCF-7 cells a valuable model for the study of the role of growth factor and growth factor receptor expression in the proliferation of breast cancer cells.

The E2 dependence for growth, antiestrogen sensitivity, and low metastatic potential of MCF-7 cells has led to the hypothesis that they represent an "early" breast cancer phenotype.[35,36] MCF-7 cells are an excellent model in which to study the process of malignant progression, because they can be subjected to appropriate endocrinologic and physiologic selective pressures for the derivation of variants with more progressed phenotypes. For example, the estrogenic requirement for tumorigenicity in immunocompromised mice has been used to select for E2-independent MCF-7 variants (see below and Chapter 22). Variants have also been selected for antiestrogen resistance, and the extent of induced cross-resistance among structurally diverse antiestrogens has been determined.

T47D

T47D cells were established by Keydar et al. from a 54-year-old patient with an infiltrating ductal carcinoma.[37] The cells express ER, PR, androgen, glucocorticoid,[37] and insulin receptors.[38] Approximately 60% of the original cell line expressed casein.[37,39] The T47D cells are perhaps most notable for their high levels of PR and their remarkable genetic and phenotypic instability.[40–43] Furthermore, these cells exhibit significant growth-regulatory responses to progestational agents.[44,45] Not surprisingly, T47D cells, and several T47D variants that have been obtained, represent the major *in vitro* human breast cancer models for the study of the antiproliferative effects of progestins and antiprogestins and the regulation of PR expression.[42–46] T47D cells also express RAR-α and RAR-γ[17] and are sensitive to the growth-inhibitory effects of retinoids and antiestrogens.[9,10]

The remarkable genetic instability of the T47D cells stands in contrast to the other ER+/PR+ human breast cancer cell lines. Subtle differences in the phenotypic characteristics of all of the ER+/PR+ cell lines are observed from laboratory to laboratory. However, these differences rarely extend to the pattern of expression of steroid hormone receptors, metastatic potential, antiestrogen responsiveness, the estrogen supplementation required for *in vivo* tumorigenicity, or metastatic potential. These are the major phenotypic characteristics of hormone-responsive breast cancer cells.[34–36] In cases in which significant divergent phenotypic responses in other ER+/PR+ cell lines are observed, these divergences are almost always the result of an imposed selective pressure (i.e., *in vivo* or *in vitro* growth in the absence of E2, selection for cytotoxic drug resistance). For example, no bona fide spontaneous ER– or PR– MCF-7 or ZR-75-1 cell lines have been described in detail, other than those generated by the imposition of selective pressures. In marked contrast, simple dilution cloning can produce T47D variants with fundamentally altered endocrine responsiveness, (e.g., ER–/PR+, estrogen unresponsiveness, and antiestrogen resistance).[40–42] Many of these variants are unstable and readily revert to the wild-type phenotype.[40,43] Other T47D variants (e.g., the ER–/PR+ $T47D_{CO}$) have been stable for many years.

ZR-75-1

ZR-75-1 cells were first described by Engel et al. in 1978.[47] They were established from an ascites that developed in a 63-year-old woman with an infiltrating ductal breast carcinoma. This patient had been receiving tamoxifen (tamoxifen citrate) for 3 months before the time when cells were removed to establish the ZR-75-1 cell line.[47] Although the ZR-75-1 cells are ER+/PR+ cells[47,48] and are growth stimulated by estrogens and inhibited by antiestrogens *in vitro*,[47,49] the patient did not respond to tamoxifen.[47] The expression of ER is up-regulated by interferons in these cells, and treatment with interferon 2α can increase sensitivity to tamoxifen.[48] ZR-75-1 cells exhibit an eightfold overexpression of c-*erb*-b2 messenger RNA (mRNA) relative to normal fibroblasts.[50] This overexpression is E2 regulated in ZR-75-1 cells, a down-regulation being associated with E2-induced cell proliferation.[51] Tamoxifen-resistant and hormone-independent ZR-75-1 variants have been described.[49,52,53]

ZR-75-1 cells also express androgen and glucocorticoid receptors[54] and are growth inhibited *in vitro* by progestins[54,55] and somatostatin analogues.[56] They express the RAR-α and RAR-γ retinoic acid isoforms[17] and are growth inhibited by several retinoids.[9,10] ZR-75-1 cells also express low levels of EGFR; altered EGFR expression is associated with tamoxifen resistance and hormone independence.[52,53] The ZR-75-1 cells appear to possess several steroid metabolism pathways.[57–59]

ESTROGEN-INDEPENDENT (ER+/PR+) AND ESTROGEN-RESPONSIVE BREAST CANCER CELL LINES

The estrogenic requirement of the MCF-7, T47D, and ZR-75-1 cells for growth *in vitro* or *in vivo* may not ade-

quately reflect the endocrine environment of many breast tumors in postmenopausal women. Several breast cancer cell lines and variants of the MCF-7, ZR-75-1, and T47D cell lines have been generated that no longer require estrogenic supplementation for growth. These continue to express ER, PR, or both, and many also retain responsiveness to endocrine agents. Several steroid-independent and steroid-responsive cell lines or variants exhibit properties that more closely resemble those of breast tumors in patients than do the steroid-dependent cell lines.

MCF-7/MIII, MCF-7/LCC1: Cells Selected for Estrogen Independence *in Vivo*

Several aspects of the MCF-7 phenotype could be considered potentially inconsistent with the human disease. For example, these cells generally do not proliferate in cell culture media devoid of estrogens and do not form proliferating tumors when orthotopically transplanted into oophorectomized immunodeficient mice. If this dependence on estrogens for growth were to exist in a tumor cell in a postmenopausal woman, the source from which the MCF-7 cells were originally obtained,[12] the cells would not give rise to detectable disease. Despite their apparent metastatic site of origin, MCF-7 cells exhibit few characteristics associated with an invasive/metastatic phenotype. Thus, we have previously suggested that the MCF-7 phenotype represents an early hypothetical breast cancer cell.[35,36]

We wished to determine if, by applying appropriate physiologic and endocrinologic selective pressures, we could obtain cells more representative of many of the ER+/PR+ cells apparent in the breast tumors of postmenopausal women. Thus, we selected MCF-7 cells by xenotransplantation into the mammary fat pads of oophorectomized, athymic, nude mice. After approximately 6 months, we obtained cells (MCF-7/MIII) that were readily reestablished *in vitro*. MCF-7/MIII cells were determined to be of MCF-7 origin by karyotype and isozyme profile analyses.[14] A further selection of MCF-7/MIII cells produced a variant designated MCF-7/LCC1, which exhibits increased metastatic potential and a shorter lag time to tumor appearance compared with MCF-7/MIII.[60,61]

We studied these cells for their respective responses to estrogens and antiestrogens both *in vitro* and *in vivo*. MCF-7/MIII and MCF-7/LCC1 cells proliferate *in vivo* and *in vitro* without estrogen supplementation[14,60] and are responsive to drugs representing each of the major classes of antiestrogens.[60,62] MCF-7/MIII cells also are inhibited by LHRH analogues.[63] Both these variants exhibit an increased metastatic potential *in vivo* and *in vitro*,[14,61] although at a much lower level than ER– cell lines.[1,64] We interpret these observations as indicating that MCF-7/MIII and MCF-7/LCC1 cells exhibit a phenotype representative of many ER+/PR+ cells present in the tumors of postmenopausal breast cancer patients. The phenotype of these cells has been reviewed in detail.[35,36,65]

MCF-7 K3: Cells Selected for Estrogen Independence *in Vitro*

MCF-7 cells also can be selected *in vitro* for their ability to proliferate in the absence of estrogenic stimulation. For example, Katzenellenbogen et al.[66] selected MCF-7 cells in cell culture media devoid of estrogens. The resultant cells (MCF-7 K3) have a phenotype that is generally similar to the MCF-7/MIII cells.[14] In our studies, these cells also form tumors in oophorectomized nude mice, but with a longer doubling time[14] and without an apparently increased metastatic potential (R. Clarke, *unpublished data*, 1992). Other, perhaps more subtle differences appear to exist. For example, the estrogen-induced gene *pS2* is constitutively expressed in MCF-7/LCC1 cells *in vitro* but retains some estrogen-inducible expression *in vivo*.[60] In the MCF-7 K3 cells, pS2 mRNA expression appears to be inhibited by E2.[67] Some evidence exists that MCF-7 K3 cells may be estrogen supersensitive,[67] and estrogen supersensitive MCF-7 cells have been previously reported by others.[68] We have no data for the cells selected *in vivo* (MCF-7/MIII, MCF-7/LCC1) that would clearly suggest that they have a supersensitive phenotype. The biological significance of the apparent differences between cells selected *in vivo* (e.g., MCF-7/MIII) and *in vitro* (e.g., MCF-7 K3) remains to be established.

MCF-7 MKS: Cells Transfected with Fibroblast Growth Factor 4

The FGFs are potent angiogenic growth factors, and several appear to be present in or secreted by human breast cancer cells, or both. Transfection of cells with FGF-4 produces cells (MCF-7 MKS) that are able to generate proliferating tumors in the absence of estrogenic stimulation.[69,70] Whereas MCF-7 cells are generally nonmetastatic, MCF-7 MKS cells produce highly vascular tumors, from which both lymphatic and lung metastases arise with a high frequency. Unlike MCF-7 cells selected for an ability to grow in a low-estrogen environment, MCF-7 MKS cells are stimulated by tamoxifen and inhibited by physiologic concentrations of estrogen.[69] The extent to which this endocrine-inverted phenotype reflects a specific phenotype in the human disease is unclear. However, these cells exhibit an endocrine response pattern similar to that of MCF-7 cells selected for *in vivo* resistance to tamoxifen (see below).

ESTROGEN-INDEPENDENT (ER±/PR±) AND ESTROGEN-UNRESPONSIVE BREAST CANCER CELL LINES

BT 20 (Mutant ER)

The BT 20 cell line is one of the older breast cancer cell lines and was established in 1958 by Lasfargues and Ozzello.[71] The cell line was obtained from a breast cancer patient with an infiltrating ductal carcinoma.[71] BT 20 cells

were initially described as being ER–/PR–,[72] but subsequently ER mRNA was detected.[73] More recently, these cells have been shown to express a novel ER mutant with an exon 5 deletion.[74] This mutation produces a protein that does not bind E2 and would appear ER– by ligand binding. Because some evidence exists that exon 5 mutant ER proteins can be transcriptionally active,[75] BT 20 cells could be considered to be ER+, hormone independent, and hormone unresponsive. These cells also express glucocorticoid receptor (GR),[72] and they have a 16-fold elevation in the mRNA levels of EGFR expression resulting from a fourfold to eightfold amplification.[50,76] BT 20 cells are tumorigenic but nonmetastatic when grown in athymic nude mice.[77]

BT474 (ER–/PR+)

BT474 cells were obtained from a solid primary infiltrating ductal carcinoma of the breast in a 60-year-old woman.[78] The cells express PR but not ER *in vitro*[78] and significantly overexpress c-Erb-b2 due to an amplification in the c-*erb*-b2 gene.[50] The level of c-Erb-b2 mRNA expression in BT474 cells is 128-fold that of normal fibroblasts, whereas EGFR is not overexpressed.[50]

$T47D_{CO}$ (ER–/PR+)

$T47D_{CO}$ cells are a variant of the ER+/PR+ T47D cells[37] and were originally described by Horwitz et al.[79] The most notable feature of these cells is their loss of ER but elevated and constitutive expression of PR.[38,45,79] The cells grow *in vitro* without E2 supplementation and are antiestrogen resistant.[79] Whereas the PR in $T47D_{CO}$ cells is E2 independent, insulin receptor expression is up-regulated by progestins, despite their growth-inhibitory effects.[45] The constitutive expression of PR in the absence of ER makes this an excellent *in vitro* model for screening progestins and antiprogestins, because no complicating requirement for E2 supplementation exists. For example, the antiproliferative effects of the progestin R5020 in T47D cells were initially thought to reflect an antiestrogenic effect.[44] Subsequent data obtained in the $T47D_{CO}$ cells demonstrated that progestins and antiprogestins exert direct growth-inhibitory effects independent of ER-mediated events.[45]

ESTROGEN-UNRESPONSIVE (ER–/PR–) BREAST CANCER CELL LINES

The majority of human breast cancer cell lines are ER–. Just as the ER+ cell lines tend to reflect the nature of ER+ tumors in breast cancer patients, the ER– cell lines exhibit characteristics similar to those of ER– breast tumors. For example, ER– tumors are generally more rapidly growing,[80] more aggressive, and exhibit a poorer prognosis[81–83]; similarly, the ER– cell lines tend to produce rapidly growing tumors in nude mice, several of which are highly invasive and some of which can produce distant metastases.[1,64] None of these cell lines responds to the antiproliferative effects of estrogens and antiestrogens unless exposed to suprapharmacologic doses. However, the absence of response to steroids does not preclude response to other noncytotoxic agents. Several ER– cell lines express retinoic acid receptors[17] and are growth inhibited by retinoids.[11,84]

MDA-MB-231 and MDA-MB-435

The MDA-MB-231 cell line is among the most widely used of the ER– human breast cancer cell lines and is frequently used as a negative control in many laboratories studying the endocrine regulation of breast cancer cell growth. The MDA-MB-231 cells were established from a 51-year-old woman with breast cancer who developed a pleural effusion. The patient had received prior endocrine therapy (oophorectomy) and cytotoxic chemotherapy (initially 5-fluorouracil and then a combination of cyclophosphamide, methotrexate, and adriamycin). She had received the combination regimen 3 weeks before removal of the fluid from which the MDA-MB-231 cell line was isolated.[85] The MDA-MB-231 cells are highly tumorigenic and can produce lung metastases from mammary fat pad tumors in nude mice.[64]

The MDA-MB-435 cells were established from a pleural effusion in a 31-year-old white woman with metastatic breast cancer.[39,85] Unlike many other patients from whom breast cancer cell lines have been obtained, this patient had received no prior systemic therapy.[86] Despite being initially described as nontumorigenic,[87] MDA-MB-435 is generally reported to be highly tumorigenic and is one of the few human breast cancer cell lines that produce lung metastases from solid tumors.[64,88] When growing as xenografts, the growth and metastases of these cells also appear responsive to several dietary manipulations.[89–91] The study of metastasis from the MDA-MB-435 cell line has been greatly simplified by the introduction of a marker (β-galactosidase) that can facilitate visualization of micrometastases.[92]

We have established an ascites variant of the MDA-MB-435 cells (MDA435/LCC6). We have routinely maintained these cells as ascites for several years and assessed their sensitivity to a series of cytotoxic drugs.[86] The ascites has a pattern of responsiveness to single agents that closely reflects the activity of these agents in breast cancer patients. The cells are also easily maintained *in vitro* and can be successfully reestablished as solid tumors or ascites in nude mice. The MDA435/LCC6 cells may provide an alternative to the L1210/P388 murine ascites (leukemia) for the screening of new agents for activity in breast cancer. The MDA435/LCC6 cells also respond to nanomolar concentrations of all-*trans*-retinoic acid, fenretinimide, and 9-*cis*-retinoic acid[11]; this response perhaps reflects the expression of the RAR-α, RAR-β, and RAR-γ isoforms by the parental MDA-MB-435 cells.[17]

Other MDA-MB Cell Lines

Up to 19 cell lines bear the MDA-MB designation; most were derived by Cailleau et al. at the M. D. Anderson Hospital and

Tumor Institute.[39] The basic characteristics and isozyme and karyotype patterns have been previously reported in some detail.[39,93] Most cell lines are ER–, with the notable exception of the MDA-MB-134 and MDA-MB-175 cell lines, which are ER+.[94] Several of these lines are of specific interest. The MDA-MB-468 cells overexpress EGFR[50]; in contrast to other breast cancer cell lines, their growth is inhibited by exogenous EGF.[95] FGF receptors are overexpressed by MDA-MB-175 cells, which are growth inhibited by FGF.[96] The MDA-MB-175 cells also exhibit an eightfold overexpression of c-*erb*-b2 relative to normal fibroblasts.[50] The MDA-MB-361 cells, which were obtained from a brain metastasis,[39] and the MDA-MB-453 cells exhibit a twofold to fourfold amplification of the c-*erb*-b2 gene, overexpressing the gene product by approximately 64-fold.[50] The external domain of c-Erb-b2 is shed from MDA-MB-361 cells and can be detected in the serum of nude mice bearing these xenografts.[97] The MDA-MB-436 cell line was derived from a 43-year-old woman with metastatic breast cancer.[39] These cells are ER– and are sensitive to several cytotoxic drugs. The MDA-MB-436 cells have been used to investigate the effects of insulin and cell-seeding density on methotrexate metabolism[98,99] and the non–ER-mediated effects of estrogens and antiestrogens on both the cytotoxicity of methotrexate[100,101] and cell membrane structure and function.[102]

SkBr3

SkBr3 cells were obtained from a pleural effusion that developed in a 43-year-old patient with a breast adenocarcinoma.[103] These cells have been widely used in the study of c-Erb-b2 expression, because they overexpress c-Erb-b2 128-fold relative to normal fibroblasts owing to a fourfold to eightfold amplification of this gene.[50] SkBr3 cells also secrete a truncated c-Erb-b2 into their cell culture medium.[104] The coexpression of EGFR and c-Erb-b2 has enabled studies into the mechanisms of EGF-induced heterodimerization.[105]

NEW BREAST CANCER CELL LINES

The following section is not intended to be exhaustive but includes several new cell lines with some novel or unusual characteristics. The methods of isolation vary across studies, but all cell lines have been confirmed to be of breast origin. When the information is available, tumorigenicity, oncogene expression, and other potentially relevant information is described.

The establishment of new cell lines is a difficult and time-consuming process. Primary cultures can be initiated with a relatively high success rate.[106,107] Biopsy material can be cultured in a manner that allows for the preferential growth of neoplastic rather than normal epithelial cells.[108] However, the proportion of these cultures that spontaneously develop into established cell lines—for example, lines that can be maintained successfully for over 50 passages—is relatively low. In a study of 135 primary breast cancers, only 10 produced cell lines. All were negative for ER.[109] The success rate appears similar when the material for culture is derived from lymph node metastases. Thus, tumor stage would not seem to be a particularly good predictor for *in vitro* establishment.[106] The very poor success rate in establishing ER+ cell lines remains problematic.

Although ER can be overexpressed in ER– breast cancer cells by transfection, the resulting cells are almost exclusively growth inhibited by physiologic concentrations of estrogens.[110] A similar phenotype is reported for overexpression of ER in the normal human breast epithelial cell line MCF10A,[111] although the antiproliferative responses to estrogens appear relatively small. Breast tumors generally contain these levels of intratumor estrogens, regardless of the patient's menopausal status.[112] Such tumors might not arise if these intratumor estrogens were growth inhibitory, or at least would not arise until this signaling was eliminated or overcome. Furthermore, the administration of exogenous estrogens—for example, estrogen-based hormone replacement therapy—generally increases breast cancer risk.[113] This is consistent with the mitogenic effects of estrogens in cells that "normally" express ER, such as MCF-7 cells, and the growth-inhibitory effects of antiestrogens. Thus, the phenotypic relevance of these transfected cells is difficult to determine. Some transfectants can regulate estrogen-responsive element (ERE)–regulated gene expression in transient transfection assays but do not regulate endogenous genes, such as the genes for PR, pS2, and cathepsin D, in response to estrogens.[111] Thus, the function of some estrogen-regulated genes may be unaffected by expression of ER.

Some breast cancers possibly may arise from cells that were normally negative for ER,[114] and cellular signaling within these populations may be different from that in cells that normally express ER and require estrogens for proliferation. Some critical genes may be regulated in a direction not seen in cells that normally express ER, perhaps accounting for the inverted—that is, estrogen-inhibited—phenotype. If the ER function is significantly different than that in cells which normally express ER, the use of these models to study signaling to estrogenic/antiestrogenic responses may require careful consideration.

One cell population has been studied that is naturally inhibited by estrogens—namely, vascular smooth muscle cells.[115–117] These cells are naturally growth inhibited by estrogens, a finding that is not surprising, as estrogen is known to reduce the risk of cardiovascular disease. One mechanism might be through the inhibition of vascular smooth muscle cell proliferation after vascular injury.[117] Although an estrogen-inhibited phenotype is not physiologically unimportant in other tissues, the extent to which it applies to breast cancer is unclear.

BRC-230

The patient from whom cells for this line were obtained was a 79-year-old woman with a metastatic, infiltrating ductal car-

cinoma. The primary tumor was ER–/PR– and exhibited a high thymidine (TdR)-labeling index.[118] The cell line, designated BRC-230, was established from surgical material obtained from the primary tumor. The cells are ER–/PR– and show no evidence of amplification or rearrangement of c-*erb*-b2, c-*myc,* or *mdr*-1. BRC-30 cells are tumorigenic in nude mice and produce carcinoembryonic antigen, CA15-3, CA19-9, and CA125.[118] Of potential interest is the pattern of chemosensitivity, which closely reflects that seen in the patient. BRC-230 cells exhibit a multiple drug–resistant phenotype and are resistant to doxorubicin hydrochloride, etoposide, idarubicin hydrochloride, mitoxantrone hydrochloride, 4'-epidoxorubicin, and 4-idroperoxy-cyclophosphamide.[118] BRC-20 cells may be useful in studying non–*MDR1*-mediated multiple drug resistance.

HMT-3909S1 and HMT-3909S8

Petersen et al.[119] have described two cell lines derived from the same primary tumor. The primary tumor was an infiltrating ductal carcinoma that arose in a 61-year-old white woman. The patient had received no prior therapy. The cell lines were established in serum-free media.[119] HMT-3909 is nontumorigenic, whereas the HMT-3909S8 line forms tumors in athymic nude mice. HMT-3909S8 cells are aneuploid and express the mesenchymal glycoprotein vimentin, keratins 8 and 18, and the MAM-6 glycoprotein. HMT-3909S1 cells express vimentin and several keratins, including keratin 18, but MAM-6 reactivity is weak and keratin 8 staining is not detected.[119] The cells are not entirely ER–, but expression appears very low. The presence of two phenotypically distinct cell lines, each derived from a single hypothetical stem cell, may provide a novel means to study progression and acquisition of phenotypic diversity in tumors.

KPL-3C

KPL-3C cells were derived from a 37-year-old Japanese woman with invasive ductal carcinoma. The patient was initially treated by radical mastectomy and subsequently received radiotherapy to the locally recurrent lesions and systemic chemoendocrine therapy. Liver metastasis and a pleural effusion were subsequently diagnosed. The cell line was established from the pleural fluid.[120] The resulting cell line (KPL-3C) is tumorigenic in athymic nude mice, with a doubling time of approximately 7 days, and produces tumors reminiscent of comedo-type intraductal neoplasms. The tumors often exhibit an area of central necrosis characterized by microcalcification. The cells express cytokeratin, epithelial membrane antigen, carcinoembryonic antigen, and CA15-3, but do not express either vimentin or c-Erb-b2. Expression of ER and PR is low, with the levels reported as approximately 15 fmol per mg protein and 14 fmol per mg protein, respectively.[120] The cell population doubling time *in vitro* is approximately 72 hours. A potentially unusual characteristic of this cell line is its secretion of parathyroid hormone–related protein. Interestingly, the patient from whom these cells were derived required treatment for humoral hypercalcemia. This cell line may be useful for studying the role of tumor-derived parathyroid hormone–related protein in humoral hypercalcemia of malignancy.[120]

LCC15-MB

The LCC15-MB cell line was established from a femoral bone metastasis. The patient was a 29-year-old woman initially diagnosed with an infiltrating ductal mammary adenocarcinoma.[121] Approximately 3 years after the initial primary tumor was diagnosed, the patient presented with acute bone metastasis. Material from the bone metastasis, a poorly differentiated adenocarcinoma lacking ER, PR, and *erb*-b2 expression, was used to establish the cell culture. The LCC15-MB cells exhibit these characteristics, although ER can be reexpressed by treatment of the LCC15-MB cells for 5 days with 5-aza-2'-deoxycytidine. LCC15-MB cells are tumorigenic in athymic nude mice and produce long-bone metastases after intracardiac injection.[121] These cells strongly express vimentin. The presence of keratin 18 mRNA has been demonstrated using assay by polymerase chain reaction, but the low overall levels of keratin protein and the lack of keratin 19 mRNA suggest that these cells have selectively lost epithelial characteristics while gaining a more mesenchymal phenotype, consistent with the epithelial to mesenchymal transition that can occur during malignant progression in breast cancer.[121] The cells are invasive in the *in vitro* Boyden chamber assay and activate matrix metalloprotease 2 after treatment with concanavalin A.[122] Significantly, LCC15-MB cells express the bone matrix protein osteopontin, and this expression is retained by subcutaneous xenografts and intraosseous metastases.[122] The LCC15-MB cells provide a unique model in which to study bone metastasis and the role of osteopontin in this process.

MFM-233

The MFM-233 cell line was derived from a pleural effusion that arose in a postmenopausal patient. The patient had not received any prior treatment and presented with a widespread grade 3 ductal carcinoma. The established cell line was designated MFM-223.[123] These cells are tumorigenic in nude mice, producing moderate to poorly differentiated adenocarcinomas. MFM-233 cells express cytokeratins 8 and 18, epithelial membrane antigen, and milk fat globulins 1 and 2. Relatively low levels of ER (5 to 10 fmol/mg protein) are expressed, but PR is not detected in the cultures and is not induced by estradiol treatment. In contrast, expression of androgen receptors is high (160 fmol/mg protein), and proliferation of the cells is inhibited by more than 0.01 nanomolar dihydrotestosterone.[123] This cell line may prove useful in the study of androgen responsiveness and signaling.

MODELS OF ACQUIRED ANTIESTROGEN RESISTANCE (ER+ CELLS)

Although acquired resistance to antiestrogens is one of the more pressing clinical problems in breast cancer, few *in vitro* models exist for the analysis of this aspect of malignant progression. The most common approaches to the isolation of antiestrogen-resistant cells use an *in vitro* selection of hormone-dependent cells against either a high single dose of antiestrogen[124] or a stepwise increase in concentrations of drug. These approaches have been widely used to generate variants of cell lines resistant to many antineoplastic agents. Several problems are encountered when this approach is applied to generate antiestrogen-resistant variants of estrogen-dependent breast cancer cells. For example, isolation of resistant clones that retain stability for several years has often been difficult. Several laboratories have reported resistant variants that revert to a sensitive phenotype with a high frequency.[49,125–127] Some cell lines alter other critical aspects of their phenotypes. The MCF-7 variant LY-2 has become nontumorigenic.[62] MCF-7 cells selected *in vivo* can become dependent on or stimulated by tamoxifen.[128,129] Cells transfected with FGFs also reverse their endocrine responsiveness, becoming stimulated by tamoxifen and inhibited by physiologic concentrations of estrogens.[70] These models may reflect a tamoxifen-withdrawal effect, although the prevalence of this response in humans is difficult to determine.

LY-2: MCF-7 Cells Selected against a Benzothiophene (LY117018) *in Vitro*

The MCF-7 variant LY-2[124] is perhaps the most stable antiestrogen-resistant variant and was generated in 1985. These cells were selected *in vitro* in an anchorage-independent (soft agar) colony assay for resistance against LY117018.[124] LY-2 cells have been demonstrated to be cross-resistant to drugs representative of the major structural classes of antiestrogens, including nafoxidine, 4-hydroxytamoxifen, and ICI 164,384.[62] In addition to exhibiting a significant shift in their dose-response relationship for antiestrogens, LY-2 cells exhibit a blunted mitogenic response to E2.[124] However, LY-2 cells have lost their ability to form proliferating tumors in oophorectomized or E2-supplemented nude mice,[62] limiting their use to *in vitro* studies.

Although the precise resistance mechanism in the LY-2 cells is unclear, these cells express ER levels approximately one-third those of their parental MCF-7 cells and have become negative for PR.[124] A reduced level of ER expression would be expected to induce resistance to all antiestrogens, because interaction with ER is likely to be the most important early event in antiestrogen function. Thus, the altered ER levels or the reduced ability to mount an estrogenic response in the LY-2 cells may explain their antiestrogen-resistance pattern. LY-2 cells still express levels of ER that would be considered high in a breast tumor biopsy.[124] Furthermore, the remaining ER appears normal—that is, it is not mutated or altered.[130] The LY-2 cells may mimic some aspects of the antiestrogen-resistance profile in patients with ER+/PR– tumors.

MCF-7 Cells Selected against Tamoxifen *in Vivo*

Several groups have generated resistance models by selecting MCF-7 xenografts growing in nude mice against tamoxifen, an approach that would appear to more closely mimic the human disease than *in vitro* selection. However, MCF-7 cells do not form proliferating tumors in castrated female mice, which have an endocrine environment similar to that of postmenopausal women.[131–133] Because the xenografts would not be proliferating, their growth could not be further suppressed; tamoxifen is generally considered a cytostatic, not cytotoxic, drug. Consequently, it might be predicted that the most efficient response to such a selective pressure would be a change in the cell's perception of tamoxifen from inhibition to the widely documented partial agonist (growth promotion at low concentrations) properties of tamoxifen.[134] Indeed, the resultant tumors exhibit a tamoxifen-stimulated/tamoxifen-dependent phenotype,[128,129] suggesting that this "inverted" phenotype reflects a sensitization to the partial agonist (estrogenic) effects of the triphenylethylenes.[134] Tamoxifen dependence is evidenced by withdrawal responses to tamoxifen that induce regression of the xenografts.[128] More recently, an ER variant has been identified that may explain these changes in responses to antiestrogens.[135,136] Jiang et al. have identified a glycine-to-valine mutation at amino acid position 400 in the ER protein. When transfected into breast cancer cells, this mutation confers a growth-inhibitory response to estrogens and a growth-stimulatory response to antiestrogens.[135,136]

A breast tumor in a patient that possessed a tamoxifen-dependent phenotype could respond to removal of tamoxifen by exhibiting a tamoxifen-withdrawal response. Withdrawal responses have been widely reported for other endocrine therapies, including high-dose estrogen and progestin treatment.[137] Whether this occurs for antiestrogens is unclear, because the incidence of tamoxifen withdrawal responses has not been clearly defined and documented. Several anecdotal and single case reports of tamoxifen withdrawal responses have been published.[138–143] Several larger studies indicate a low incidence of tamoxifen withdrawal responses.[144,145] Thus, the data from these models may be predicting a response yet to be clearly demonstrated in the clinic or demonstrating an experimental artifact. Should these models be correct, the potential for a significant incidence of tamoxifen withdrawal responses could indicate an important, and potentially underestimated, clinical response pattern.

Selection of Hormone-Independent but Hormone-Responsive Cells against 4-Hydroxytamoxifen

Rather than use hormone-dependent cells and risk a loss of tumorigenicity (e.g., the LY-2 phenotype) or select *in vivo* using hormone-dependent cells and obtain a tamoxifen-stimulated phenotype, we hypothesized that cells already hormone independent and responsive might provide a more appropriate starting point for the generation of resistant variants. These cells already proliferate in the absence of estrogenic stimulation both *in vivo* and *in vitro.* To eliminate species-specific metabolic differences between rodents and humans, we chose to perform a stepwise selection of the MCF-7/LCC1 cells *in vitro* against the potent tamoxifen metabolite 4-hydroxytamoxifen. We obtained a stable resistant population designated MCF-7/LCC2. These cells are resistant to tamoxifen when growing either *in vitro* or as xenografts in nude mice[146] and have remained stably resistant in the absence of selective pressure.

We determined the likely cross-resistance profile of these cells by assessing their *in vitro* growth response to steroidal antiestrogens. Although resistant to tamoxifen, MCF-7/LCC2 cells are not cross-resistant to either ICI 182,780 or ICI 164,384.[146,147] This response pattern suggested that some patients who initially respond to tamoxifen but ultimately relapse may retain the ability to respond to a steroidal antiestrogen. Subsequently, this resistance pattern has been observed in preliminary data from a Phase I trial of ICI 182,780 in heavily tamoxifen pretreated patients.[148] These data indicate that, as predicted by the MCF-7/LCC2 phenotype, patients that ultimately relapse on tamoxifen can obtain responses to a subsequent steroidal antiestrogen treatment. Thus, an *in vitro* observation correctly predicted a subsequent pattern of response in breast cancer patients. These data suggest that the clinical responses to ICI 182,780 probably represent a genuine direct antitumor effect, rather than a possible tamoxifen withdrawal response, and suggest that the MCF-7/LCC2 phenotype is not merely an *in vitro* artifact. The relevance of these cells and their phenotypes has been reviewed.[36,65]

Cells Selected for Resistance to Steroidal Antiestrogens

MCF-7/LCC1 cells selected against the steroidal antiestrogen ICI 182,780 have been isolated and characterized. An *in vitro* stepwise selection was used similar to that used to generate the MCF-7/LCC2 cells. The stable ICI 182,780–resistant population was designated MCF-7/LCC9. MCF-7/LCC9 cells are resistant to ICI 182,780 *in vitro* and *in vivo.*[149] Our data indicate that these cells exhibit cross-resistance to tamoxifen, even though the cells have not been exposed to a triphenylethylene antiestrogen. If correct, this pattern of *in vitro* resistance would suggest that patients may be better served if treated initially with tamoxifen and subsequently with a steroidal antiestrogen, rather than vice versa. The validity of this prediction remains to be tested in patients.

MODELS FOR STUDYING MULTIPLE-DRUG RESISTANCE (*MDR1*/GP170)

Many breast tumors are often initially responsive to cytotoxic chemotherapy. Almost all develop a multiple drug–resistant phenotype, however, and this is ultimately responsible for the failure of current cytotoxic regimens.[34] Acquired resistance is frequently associated with expression of the *MDR1* gene and its gp170 glycoprotein product. The level and incidence of detectable *MDR1*/gp170 expression is significantly higher in the tumors of treated versus untreated breast cancer patients[150–152] and correlates with *in vitro* resistance to cytotoxic drugs.[152–154] Several *in vitro* models have been established with which to screen for new agents that can reverse this form of multiple drug resistance.

Cells Selected for Resistance against Adriamycin

Cell lines selected *in vitro* for resistance to adriamycin frequently overexpress gp170, often as a result of amplification of the *MDR1* gene. Among the most widely used cell lines are the MCF-7^{ADR} line[155] and the HeLa (ovarian carcinoma) variant KbV series.[156] The origin of the MCF-7^{ADR} cells, as used in the current National Institutes of Health (NIH) drug-screening program, has been questioned. The MCF-7 origin of these cells could not readily be determined, and the cell line has been redesignated NCI/ADR-RES.[157] The extent to which these cells can be used as a specific model of multiple drug–resistant breast cancer is unclear.

One problem with cells selected *in vitro* is that they frequently acquire multiple drug–resistance mechanisms. For example, we have demonstrated that NCI/ADR-RES (MCF-7^{ADR}) cells, but not *MDR1*-transduced MCF-7 cells (CL 10.3), are cross-resistant to tumor necrosis factor.[158] Because both adriamycin and tumor necrosis factor can inhibit cells by the generation of free radicals,[159,160] this cross-resistance in NCI/ADR-RES cells strongly suggests the presence of adriamycin-resistant mechanisms in addition to gp170, including altered expression of manganous superoxide dismutase.[158] Indeed, NCI/ADR-RES cells also exhibit increased glutathione transferase and topoisomerase II activities[161,162] and have become estrogen independent and antiestrogen resistant due to their loss of steroid hormone receptor expression.[155]

The complexity of the resistance phenotype in these cells may explain why the gp170-reversing potency of isomers of flupenthixol identified in NCI/ADR-RES (MCF-7^{ADR}) cells could not be confirmed in *MDR1*-transfected NIH 3T3 cells,[163] which suggests a non–gp170-mediated mechanism. Although NCI/ADR-RES cells are clearly of considerable use for screening new resistance-modifying agents and combinations, their use for detailed mechanistic studies of resistance reversal may be limited. These cells are widely used and very well characterized, however, and they provide an important benchmark for comparing data among different studies.

Cells Transduced with the *MDR1* Gene

To obtain cells in cases in which gp170 is the major multiple drug–resistance mechanism, a cloned, E2-dependent, MCF-7 human breast cancer subline was transduced with a retroviral vector directing the constitutive expression of the *MDR1* gene.[164] After selection in the presence of the gp170 substrate colchicine, cell populations (MCF-7^{MDR1}) were isolated, and their ability to produce gp170 was determined by radioimmunoprecipitation.[164] In this study, one of the MCF-7^{MDR1} clones designated CL 10.3 was used. Transduced cells express high levels of a 170-kd glycoprotein exhibiting immunoreactivity with specific anti-gp170 antibodies. Immunoreactivity is not detected in either the parental MCF-7 cells or MCF-7 cells transduced with a control pSV2neo vector. The level of expression of MCF-7^{MDR1} cells is estimated to be within twofold to threefold of that expressed by the adriamycin-selected NCI/ADR-RES cells.[163] The function of the expressed glycoprotein was confirmed by determining the sensitivity of parental and MCF-7^{MDR1} cells to a gp170 substrate (adriamycin) and to a non-gp170 substrate (gossypol).[164,165] Transduced cells have a tenfold greater IC_{50} for adriamycin, whereas sensitivity to gossypol is equivalent in both parental and transduced cells.[164] A similar relationship has been observed for colchicine and the non-gp170 substrate methotrexate (R. Clarke and F. Leonessa, *unpublished observations*, 1990).

The increase in resistance exhibited by the transduced MCF-7^{MDR1} cells would be expected to be sufficient to induce clinical resistance in tumors. Perturbations in energy metabolism in the MCF-7^{MDR1} cells have also been observed that are not present in the parental cells.[165] *MDR1*-transduced cells retain ER and PR expression and sensitivity to the triphenylethylene antiestrogen 4-hydroxytamoxifen.[164] Expression of the estrogen-inducible pS2 and EGFR genes are similar in parental and MCF-7^{MDR1} cells.[164] EGFR is upregulated, and pS2 expression is lost in NCI/ADR-RES cells.[155,164] The data indicate that overexpression of the *MDR1* gene alone confers a multiple drug–resistance phenotype but does not result in either cross-resistance to antiestrogens or a loss of steroid hormone receptor expression.[164]

The ascites variant MDA435/LCC6 of the ER– breast cancer cell line MDA-MB-435 has been transduced with the *MDR1* complementary DNA. The MDA435/LCC6 cells appear to retain the major characteristics of their parental cells; that is, they are ER–, highly tumorigenic, invasive, and metastatic.[166] When the cells are grown as an ascites, the mice become moribund within a reproducible time (approximately 30 days) and exhibit a pattern of responses to established cytotoxic drugs that closely reflects the activity of the agents when administered as single agents to previously untreated breast cancer patients.[166] The *MDR1*-transduced cells (MDA435/LCC6^{MDR1}) provide an ER– model for comparison with the ER+ MCF-7^{MDR1} CL 10.3 cells. The ascites variants provide an alternative to the murine leukemia ascites models (e.g., L1210, P388) for screening gp170-reversing agents.[166]

NORMAL BREAST EPITHELIAL CELLS AND THEIR DERIVATIVES

Culturing Normal Breast Epithelial Cells *in Vitro*

Major advances have been made in the culture of normal mammary cells from both humans and rodents. Not all of the approaches can be discussed in detail here, but several excellent reviews are available.[167,168] Of particular importance are models that allow for the coculturing of stromal and epithelial cells, because the interactions among these populations appear critical for normal glandular development and function. The approaches reviewed by Ip and Darcy[167] demonstrate the ability of cells maintained *in vitro* to complete a phenotypically normal lobuloalveolar development. These structures secrete milk proteins in response to appropriate hormonal stimuli and undergo an apparent involution on hormone withdrawal.[167] The culture techniques have been optimized for, and widely applied to, both human and rodent mammary cells.[167,168] For example, normal human epithelial cells proliferate and differentiate in a three-dimensional sponge-gel matrix culture system.[169]

These approaches require the isolation of viable epithelial or stromal cells from solid tissues. Many investigators appear to use one of several collagenase-based disaggregation methods,[167,170] but explant, organ culture, and organoid approaches also are successful.[106,167,171] The most effective approaches generally differ from the standard cell culture techniques used to propagate and study breast cancer cells, primarily in the provision of a three dimensional environment and the inclusion of stromal cells.[167,168] The success rate in establishing primary cultures of both normal and neoplastic mammary tissues has increased significantly. Even relatively simple approaches can produce short-term cultures on plastic with good reproducibility. For example, Volpi et al. have reported success rates of 83% for primary human breast cancers and 78% for normal breast tissue.[106]

Several specialized cell culture media have been generated that have greatly increased the success rate for establishing primary cultures. In general, these are semi-synthetic media that contain little or no serum, have low levels of Ca^{2+}, and are supplemented with various hormones, growth factors, and chemically undefined ingredients, such as conditioned cell culture media and bovine pituitary extract.[106,172–175]

Although these cells represent primary cultures—that is, they have a finite life span *in vitro*—they may be immortalized by treating them with carcinogens and transformed by inducing an overexpression of several oncogenes. As with the neoplastic breast cell lines, several caveats should be borne in mind. For example, the primary and immortalized cells are adapted to *in vitro* growth, and some of their

expressed (or repressed) characteristics may be more closely associated with this adaptation than their normal *in vivo* function. However, this concern most likely is minimized when three-dimensional culture matrices are used and stromal cells are included. Immortalized cells are continually proliferating, a state quite different from the resting tissues from which their parental cells were derived.

All the available cell lines established from normal mammary cells are ER–. ER has been introduced into several normal human breast epithelial cell lines. However, the resultant phenotype is growth inhibited by estradiol.[176] A similar phenotype occurs when breast cancer cells are transfected with ER.[110] This E2-inhibited phenotype appears counterintuitive, because estrogens are generally considered mitogens in both normal and neoplastic breast tissues. Nevertheless, these potentially "normal" cells are important models that provide the opportunity to study aspects of the biology of normal mammary epithelial cells, to identify agents that may contribute to the malignant transformation of normal mammary cells, and to determine phenotypic and genotypic perturbations associated with this process.

Benzo(*a*)Pyrene-Immortalized 184 and B5 Lineages from Reduction Mammoplasties

Stampfer and Bartley[173] have successfully established primary organoid cultures from normal reduction mammoplasty tissues. The source tissues for these cultures were essentially resting, in that they were not obtained during a functional or active period, such as early pregnancy, lactation, or involution.[173] These cells can readily be immortalized by treatment with benzo(*a*)pyrene (e.g., 184 cells). Immortalized normal mammary epithelial cells can exhibit evidence of their breast epithelial origin. For example, the cells are clearly epithelial,[172] express several human milk fat globulin antigens, and synthesize α-lactalbumin and β-casein.[173] Although immortalized—that is, they can be maintained continuously *in vitro*—these cells are not considered transformed according to several criteria, including their inability to form tumors in nude mice or significant anchorage-independent growth.[173]

Transformation of Immortalized Human Mammary Epithelial Cells with Oncogenes

The introduction of viral or cellular oncogenes into benzo(*a*)pyrene-immortalized human mammary epithelial cell lines results in a stepwise progression from a normal to a malignant phenotype.[173,177–179] Two distinct immortalized lineages (184AIN4 and 184B5) have been characterized after transformation by several viral oncogenes. Tumorigenicity in nude mice is observed after infection of the benzo(*a*)pyrene-immortalized 184A1N4 subline with v-Ha-*ras* (A1N4-H), but not v-*mos* (A1N4-M), c-*myc* (A1N4-myc), or *SV40T* (A1N4-T; after limited passaging of cells).[177] Although they are nontumorigenic, v-*mos*-, c-*myc*-, and *SV40T*-transformed cells do exhibit phenotypic transformation and autonomy from growth factors *in vitro* to varying degrees. Combination of v-Ha-*ras* with v-*mos* (A1N4-MH) or *SV40T* (A1N4-TH) resulted in highly malignant and metastatic tumors in the nude mouse.[177,178] Consistent with the effects of v-Ha-*ras* on the A1N4 cell line, infection of the 184B5 subline with v-Ki-*ras* (B5kTu cells) also confers tumorigenicity in nude mice.[173]

MCF-10A, MCF10AT, and MCF10AT1

Soule et al.[175] have described a spontaneously immortalized "normal" human breast epithelial cell line (MCF-10). The cells were isolated from mastectomy tissue obtained from a 36-year-old premenopausal woman with benign fibrocystic disease. After 849 days in culture, a population designated MCF-10A was established. These cells exhibit a stable t(3;9)(3p13;9p22) translocation.[175] The MCF-10A cells resemble luminal epithelial cells rather than myoepithelial cells, and express antigens for several keratins and epithelial sialomucins.[180] The cells are nontumorigenic in nude mice and do not exhibit anchorage-independent growth.[175] These cells have also been used to assess the transforming ability of several oncogenes. Transfection with the ER gene was not sufficient to produce transformation.[111] MCF-10 cells cotransfected with the *erb*-b2 and Ha-*ras* oncogenes (MCF-10A HE) exhibited a substantial increase in soft agar clonogenicity but lacked significant tumorigenicity in nude mice.[181]

Transformation with the Ha-*ras* oncogene alone (MCF10AT) caused an increase in clonogenicity chemotaxis and degradation of basement membrane *in vitro*.[182] However, the cells are poorly tumorigenic in nude mice. Small, palpable nodules do arise, and these can persist.[183] Sporadic progression to carcinoma was observed, and cells from one of these was reestablished *in vitro* (MCF10AT1). MCF10T1 cells can produce simple ducts when embedded in Matrigel and transplanted into immunodeficient mice.[183,184] Up to 25% progress to invasive carcinoma.[184] Expression of c-*erb*-b2 was detected in 50% of the atypical hyperplasias and 78% of the invasive adenocarcinomas.[185] These cells provide an important and unique model for the progression from atypical hyperplasia to carcinoma.

HBL-100

The HBL-100 cell line is comprised of cells obtained from an early lactation sample.[186] The cells have been described as being of myoepithelial origin.[187] The donor was an apparently healthy woman and had no evidence of breast lesions.[186,188] The cells can form colonies in soft agar, however, and are aneuploid.[186] Although early passage cells are nontumorigenic, HBL-100 cells become tumorigenic after repeated passage *in vitro*, generally around passage 70.[187,189–191] This appears to be associated with the acquisition of specific marker chromosomes[192] and alterations in microfilament and microtubules,[189] and overexpression of

an 89-kd heat shock protein in late passage.[193] Although some HBL-100 cell stocks contain Mason-Pfizer monkey virus,[194] the ability to acquire a transformed phenotype appears to be related to the incorporation of *SV40* sequences into the genome.[188,190,195,196] The cells bind and respond to glucocorticoids and EGF,[197] and express functional β-adrenergic receptors[198] and the IGF-I receptor.[199] Although HBL-100 cells are negative for ER and prolactin receptors,[200] they may require activity of their FGF-2 autocrine loop for maximal proliferation.[201]

Although these cells are derived from an apparently normal donor, it is not entirely clear that they can be considered to represent normal mammary cells. Care must be exercised when selecting HBL-100 cells as a model of normal breast cells. For such a model, their use should probably be restricted to cultures of as early a passage as possible, and almost certainly to cells of passages earlier than 70. Under other circumstances, HBL-100 cells provide a potentially useful model in which to study transformation and progression.

IN VITRO MODELS FOR STUDYING INVASION AND METASTASIS

To metastasize effectively, cells must accomplish a complex compendium of activities, including escape from the primary lesion, avoidance of immune surveillance, and penetration into normal tissue at distant sites.[202] Invasion of extracellular matrices occurs repeatedly in this process, and basement membrane invasion, in particular, has received considerable attention.[203] The loss of basement membrane at the parenchymal-mesenchymal interface of locally invasive tumors has been closely associated with metastatic dissemination.[204–206] The uniformity of basement membrane composition and structure suggests that the molecular mechanisms involved in basement membrane recognition, attachment, degradation, and traversal may yield novel targets for cancer therapy. Several *in vitro* models have been used to study the process of basement membrane invasion and its relationship to malignant progression.

In Vitro Assays for Invasive Potential

The development of Matrigel, a reconstituted basement membrane extract from the EHS (Engelbreth-Holm-Swarm) sarcoma,[207] has been instrumental in facilitating compositional and functional analyses of basement membranes. Matrigel is liquid at 4°C, so that various manipulations are possible before it sets into a homogeneous gel at 37°C. Matrigel contains the major basement membrane components, including laminin, collagen type IV, and heparan sulfate proteoglycan. Matrigel has been used in two different assays to examine *in vitro* invasiveness of breast cancer cells.[208]

The Boyden chamber chemoinvasion assay[209] compares the ability of cells to traverse a Matrigel-coated polycarbonate filter as they migrate toward different chemical attractants. Invasive cells, stained on the lower filter surface, can be quantitated either by image analysis or crystal violet staining.[210] Although the assay was originally developed in modified blind-well Boyden chambers, two-compartment chamber systems, such as Transwell from Gibco (Rockville, MD) and Bio-Coat wells from Collaborative Research/ Beckton Dickinson Labware (Franklin Lakes, NJ), have been used successfully. Other adaptations include prelabeling of the cells with either a nontoxic fluorescent dye or radioactive agent to facilitate quantitation of invaded cells.

The ability of cells to form invasive colonies when embedded in a three-dimensional gel of Matrigel is compared qualitatively in the Matrigel-outgrowth assay.[209,210] Cells dispersed in a three-dimensional layer of Matrigel are examined after culture for 2 to 10 days. Although dispersal of single cells throughout the upper layer of Matrigel provides the most stringent test for invasive outgrowth, characteristic morphologies can be achieved more rapidly with cells sandwiched between two layers of Matrigel or simply plated on top of Matrigel.

The presence or lack of ER is an important determinant of both prognosis and choice of treatment of breast cancer. The hormone-responsive or hormone-dependent breast cancer cell lines represent a model system for the analysis of hormonal influences on the invasive process. Effects of estrogens, antiestrogens, and progestins on the *in vitro* invasiveness of steroid-dependent and steroid-responsive human breast cancer cells have been reviewed.[211–214] In addition, progression to hormone independence has implications for invasiveness and metastasis. For example, MCF-7/MIII and MCF-7/LCC1 cells have been shown to acquire an increased metastatic potential as they become estrogen independent.[61] This increased metastatic potential is reflected in increased activity in the Boyden chamber[62] (but not in Matrigel outgrowth assays), increased local invasiveness *in vivo*, and an ability to produce occasional distant metastases in nude mice.[61]

To examine the hypothesis that ER– human breast cancer cell lines are constitutively more invasive than their ER+ counterparts, a large number of the human breast cancer cell lines described above have been examined for invasiveness in the Boyden chamber assay *in vitro* and for metastatic potential in the nude mouse. These studies clearly indicate that the majority of ER– cell lines are inherently more aggressive than ER+ cells both *in vitro* and *in vivo*.[1,208] Because the incidence of distant metastases is significantly lower and less reproducible than that observed in the MDA-MB-435 cells, the MCF-7/MIII and MCF-7/LCC1 cells appear to represent a phenotype intermediate between the poorly invasive MCF-7 and the metastatic MDA-MB-435.[61]

Cell Lines and the Epithelial-to-Mesenchymal Transition

An emerging development in progression studies for breast carcinoma is the immunocytochemical analysis of markers characteristic of epithelial or mesenchymal phenotypes. The

mesenchymal intermediate filament glycoprotein vimentin (VIM) has been associated with lack of ER, high growth fraction, and poor nuclear grade in human breast cancer.[215–219] VIM expression in the tumor component is indicative of an epithelial-to-mesenchymal transition, which may occur during the process of malignant progression. Consistent with this notion, the epithelial marker E-cadherin, a homotypic cell adhesion molecule, is lost from more aggressive tumors.[220–222] Loss of E-cadherin and acquisition of VIM expression are events that characterize the epithelial-to-mesenchymal transition that occurs during embryogenesis.[223]

To begin to address this hypothesis, the invasiveness of epithelial-like (VIM–) and mesenchymal-like (VIM+) human breast cancer cells has been compared in the Boyden chamber and Matrigel outgrowth assays. Irrespective of hormone responsiveness, VIM+ cells exhibited significantly higher levels of both *in vitro* invasiveness and metastatic potential than did the VIM– negative group.[1,208] VIM expression was not detected in cells containing ER and was present in only some of the cell lines lacking ER, whereas E-cadherin was expressed functionally in all cell lines expressing ER as well as some that had lost ER expression and did not express VIM. These data suggest that the loss of E-cadherin expression is not linked to hormone independence but occurs earlier than VIM expression in the progression cascade. VIM expression also appears to be downstream of hormone independence.

E-cadherin expression, indicative of an epithelial phenotype, is associated with a compacted spherical morphology in VIM– cell lines when cultured in Matrigel.[224] E-cadherin is not present in any VIM+ cell lines. Absence of both E-cadherin and VIM is associated with a noninvasive cluster-type morphology. The NCI/ADR-RES cell line, derived from the MCF-7 cells by stepwise selection for increasing resistance to the drug adriamycin (see the previous section, Cells Selected for Resistance against Adriamycin), is interesting in this regard. Thought to be derived from the E-cadherin+/ER+/VIM– MCF-7 phenotype, this subline has lost ER and E-cadherin, gained VIM expression, and become significantly more invasive. Examination of additional adriamycin-resistant and vinblastine sulfate–resistant variants of the MCF-7 and ZR-75-1B cell lines shows that most, but not all, drug-resistant sublines expressed VIM.[225] Understanding of the relationships among drug resistance, VIM expression, and invasiveness may provide important clues for the optimization of chemotherapy for breast tumors.

The MCF-7/MIII and MCF-7/LCC1 variants retain ER and uvomorulin (UVO) expression, generally lack VIM expression, and exhibit somewhat lower levels of invasiveness than the VIM+ human breast cancer cells.[1,208] These observations support the hypothesis that these MCF-7 variants represent an intermediate point in the metastatic progression of breast cancer. The metastatic potential of the MCF-7/MIII and MCF-7/LCC1 cells, compared with the ER–/VIM– cell lines (e.g., MDA-MB-468),[61] however, suggests that metastatic potential may also develop independently of an event similar to the epithelial-to-mesenchymal transition.[226]

Oncogene Expression and *in Vitro* Metastatic Potential

The effects of oncogenes on mammary cell invasiveness have been examined. The *ras* oncogene is perhaps best studied and can induce the invasive phenotype in a variety of both human and rodent epithelial systems. Transfection of human bronchial epithelial cells transfected with v-Ha-*ras* increased both invasiveness *in vitro* and metastatic potential in the nude mouse.[227,228] In NIH/3T3 cells, transfection with either v-Ha-*ras* or genomic DNA containing various forms of activated *ras* also resulted in increased invasiveness across the amniotic membrane *in vitro* and metastatic dissemination *in vivo*.[229]

MCF-7 cells transfected with v-Ha-*ras* show increased *in vitro* invasiveness of Matrigel, increased migration potential, and increased recognition of laminin,[230] but no apparent increases in metastatic potential in nude mice.[231,232] Although both of the 184 sublines (A1N4 and B5) are transformed to a tumorigenic phenotype by expression of *ras* alone, only the initially more invasive A1N4 cells respond to *ras* transformation with increased invasiveness. The refractory nature of the 184B5 cells to *ras*-induced effects on invasiveness, despite the induction of tumorigenicity, suggests a possible lineage specificity for this response and begins to dissociate *ras* effects on tumorigenicity from invasiveness.

Differential induction of metastatic potential by v-Ha-*ras* has been previously reported.[182,231–233] Detailed analysis of a highly stable rat mammary subclone after *ras* transduction implicated rapid phenotypic diversification rather than direct effects on a cascade of metastasis-effector genes.[234] No changes similar to the epithelial-to-mesenchymal transition were seen after *ras* transformation of the A1N4 or B5 cells[213] or in the *ras*-transfected MCF-7 cells.[211] In contrast, combined transformation of 184A1N4–immortalized human mammary epithelial cells with v-Ha-*ras* and either *SV40T* or v-*mos* induces a VIM+, invasive phenotype indicative of the epithelial-to-mesenchymal transition event.[213]

The chemotactic activity and invasive property of the MCF-10A cells cotransfected with both Ha-*ras* and *erb*-b2 (MCF-10 HE cells) has also been further investigated using the Matrigel-based assays. MCF-10A HE cells showed tenfold higher invasiveness than the nontransfected cells, formed branching colonies in Matrigel, and showed a high cloning efficiency in soft agar (Thompson et al., *unpublished data*, 1999). These attributes are indicative of a VIM+ phenotype resulting from an event similar to the epithelial-to-mesenchymal transition.

CONCLUDING COMMENTS ON *IN VITRO* MODELS

Human breast cancer cell lines growing *in vitro* and as human xenografts *in vivo* have a central role in most basic and preclinical breast cancer research. They have been widely used to investigate the cellular and molecular events associated with endocrine responsiveness, malignant progression, invasiveness, and metastatic potential. With the increasing

restrictions being imposed on the use of vertebrate animals, and the relatively limited number of species that develop spontaneous mammary carcinomas, the emphasis on the *in vitro* use of human breast cancer cell lines seems likely to increase in the coming decades. Consequently, the introduction of additional representative human breast cancer cell lines, particularly hormone-responsive lines, and the realistic assessment and acknowledgment of the caveats associated with the use of *in vitro* models are critical.

Some Caveats Regarding the Use of *in Vitro* Models

Despite their widespread use and the considerable data arising from it, *in vitro* models have several potential limitations. Relatively few well-characterized ER+ cell lines exist. Although these cell lines tend to exhibit comparable estrogenic responses in the end points most widely applied, the extent to which these observations may be applied to all ER+ human breast tumors is unclear. Certainly, many of the most important attributes, such as growth inhibition by antiestrogens, are likely to closely reflect the human disease. In cases in which responses differ markedly from predicted or observed responses in humans, such as growth inhibition by physiologic levels of E2, a greater degree of caution is clearly warranted. A clear deficit in the range of breast cancer cell lines currently available is the relatively small number of ER+ and E2-responsive cell lines.

The majority of steroid-responsive cell lines have been established, not from solid tumors, but from malignant effusions. Although such effusions can occur with a 26% to 49% frequency in breast cancer patients,[235,236] they may not be fully representative of all solid tumors. Despite the likely metastatic origin of these cells, the ER+ cell lines from these sites are rarely metastatic *in vivo*, even in severely immunocompromised animals (see Chapter 22).

The most widely used cell lines have now been in use since the 1970s. Subtle changes may have been acquired during this period, and these may not adequately reflect changes that occur in human tumors in patients. Because the cells are clearly adapted to grow *in vitro*, the perturbations that have conferred this ability also may not occur in patients' tumors. Human breast tumors are highly heterogeneous and contain many subpopulations of cells with different phenotypic characteristics, including both ER+ and ER– cells.[237] In contrast, breast cancer cell lines are relatively homogeneous. This can be viewed either as an advantage or as a disadvantage. Although responses in a cell line may not fully reflect the response of a complex human tumor, they do provide the ability to study, in considerable detail and complexity, the responses of cells representative of tumor subpopulations.

In principle, cell lines are like any other experimental model. When their limitations are openly acknowledged and appropriately considered in experimental design and data analysis, they can provide useful and important tools. Otherwise, the risk exists of overinterpretation of data or the pursuit of a potential *in vitro* artifact. As a generalization, those observations from *in vitro* models that clearly reflect the human disease are more likely to reflect real events and lead to new insights into mechanistic processes. When these models are used to generate hypotheses for future testing in humans, the validity of the observation awaits completion of the human trials. Thus, major strengths of *in vitro* models include the ability (a) to study a specific cell type and elucidate the mechanism of its response to agents at the cellular and molecular level, (b) to identify mechanistic processes by comparing related cells with different phenotypic characteristics, (c) to facilitate further hypothesis generation and testing *in vivo* when cells are grown as xenografts, and (d) to generate hypotheses for testing in the ultimate model, the human being.

Establishment and Characterization of New Breast Cancer Cell Lines and Variants

Despite the presence of a number of breast cancer cell lines[2] and the emergence of new cell lines,[238] only three of those that are in common or widespread usage (the parental MCF-7, T47D, and ZR-75-1) are clearly estrogen dependent or estrogen responsive. Few of the established cell lines are metastatic in nude mice, and those that metastasize with a high frequency are generally ER–. Thus, new breast cell lines from malignant, solid metastatic (e.g., bone, lung), and normal tissues—specifically, steroid-hormone-responsive cell lines—are needed. Unfortunately, breast cancer cells from patients' tumors are notoriously difficult to establish *in vitro*.[239–241] The take rate for xenografts of tumor tissue in immunocompromised rodents also is relatively poor, although it is generally higher than that for direct *in vitro* growth.[242] Also, a well-characterized panel of nonmalignant breast epithelial cell lines that could be used for comparative studies is notably absent. Of those "normal" cell lines available, none is steroid hormone responsive.[172,173]

New breast cancer cell lines require careful description and characterization. When possible, the characteristics and history of the patient (e.g., age, sex, race, treatment) and the known characteristics of the tumor (e.g., histopathologic diagnosis, tumor grade, nodal status, ER and PR expression, S-phase/proliferativeness, and any other pertinent information) should be provided. The human origin of the tissues should be confirmed, and a karyotype and isoenzyme profile should be reported. Typical polymorphic enzymes analyzed include lactate dehydrogenase, glucose-6-phosphate dehydrogenase (EC 1.1.1.49), phosphoglucomutase-1 (EC 2.7.5.1), phosphoglucomutase-3 (EC 2.7.5.1), esterase D (EC 3.1.1.1), mitochondrial malic enzyme (EC 1.1.1.40), adenylate kinase (EC 2.7.4.3), and glyoxalase (EC 4.4.1.5).[14] These data are particularly useful in confirming the origin of a variant as being derived from its parent and in excluding contamination of a new cell line with cells of an established cell line. To this end, we have routinely used the services currently provided by Dr. Joseph Kaplan through the Cell Culture Laboratory of the Children's Hospital of Michigan in Detroit. This facility is currently maintained under National Cancer Institute contract to provide these services.

The general characteristics of the new cell line or variant should be clearly provided. This includes *in vitro* growth characteristics (e.g., culture conditions, cell doubling time, split ratio, and the passage number at which these data were obtained) and the hormone-receptor profile and endocrine responsiveness. A description of the morphology of the cells (e.g., ultrastructural analyses), particularly one that addresses tissue origin or evidence of differentiated function (e.g., secretory activity, production of milk proteins), is strongly encouraged.[2]

The tumorigenicity of a new cell line should be determined in at least one immunocompromised rodent model (preferably several) and reported. Different immunocompromised mice strains can exhibit different abilities to support xenografts, and the model in which tumorigenicity is assessed should be clearly indicated. The inability to form tumors in athymic nude mice may not indicate that the cells are nontumorigenic in other strains (e.g., *scid*; see Chapter 22). The histology of any arising tumors should be compared with that of the original tumor when possible. The presence of any metastases and their histology also should be documented. When available, any other pertinent data (e.g., oncogene expression or amplification) should also be provided.

The designation of a cell line or variant should follow the guidelines of the Tissue Culture Association and should reflect both the tissue of origin and the laboratory in which it was established.[243] For our variant cells, we have chosen to use a designation that appends our institution to that of the original (parental) cell line (e.g., MCF-7/LCC1).

Cell Culture Conditions

The choice of culture conditions often can inadvertently influence the experimental outcome. For example, for many years, insulin was routinely added to the cell culture media used to maintain breast cancer cells. Although insulin is a potent mitogen for many of these cells, it does not appear to be required for growth *in vitro* in serum-supplemented media for most human breast cancer cell lines. Insulin can down-regulate ER expression, however.[244] Insulin and EGF have been added to serum-free media for breast cancer cells,[245] and both can influence the growth-inhibitory effects of antiestrogens.[246,247] Phenol red is widely used as a pH indicator in cell culture media. A contaminant in phenol red is known to be estrogenic, and this activity can alter both the growth and antiestrogen responsiveness.[248,249]

Serum contains various growth factors and steroid hormones. The steroids can be readily removed by treatment with charcoal-coated dextran.[250] MCF-7 cells also can use the steroid sulfates present in serum.[251] These steroid sulfates can be removed by prior treatment with sulfatase.[250] The growth factors and other proteins can be chemically inactivated to produce a growth factor–free serum.[252] The concentration of serum used also can be important. The dose-response relationship for antiestrogens is altered significantly by serum concentration.[253] We have found a final concentration of either 5% fetal calf serum or 5% charcoal/dextran-stripped serum to provide appropriate *in vitro* growth characteristics for most human breast cancer cell lines. The concentrations of steroids, growth factors, and other constituents in serum may vary considerably from batch to batch.

Estrogens remain within cells for several days,[254] and when stripping cells of endogenous estrogens, one must often thoroughly wash cell monolayers and maintain cells for several days in the absence of estrogens. We routinely wash cells at least three times with phenol red–free media supplemented with 5% dextran/charcoal-stripped serum and maintain the washed monolayers in this medium for a further 3 to 5 days to ensure adequate removal of endogenous steroids.[14,62]

The cell-seeding density also can have considerable effects on cellular growth and metabolism. Cells seeded at different densities have previously been demonstrated to exhibit both different cell population doubling times and differences in methotrexate poly-γ-glutamate formation.[99] In many instances, one must closely control for seeding density, proliferative capacity, confluence, and serum batch. The handling of cells, including duration of trypsinization and time at room temperature during passage or treatment, may also be important. For some cell lines, a trypsinized cell suspension must be passed through a sterile needle to generate a single cell suspension.

These examples indicate the general importance of closely controlling cell culture conditions. The reader is referred to several excellent books on tissue culture techniques for a more detailed description of cell culture procedures.[255–257]

SUMMARY

The development of stable cell lines derived from malignant and normal human breast tissue has been of considerable use in breast cancer research, and such cell lines continue to occupy a central position in basic breast cancer research. These cell lines provide the ability to conduct studies that could not easily be performed in experimental animals or human beings. The ease of use, relatively low cost of maintenance, general reproducibility of phenotype, and ability to mimic properties seen in tumors in patients are considerable advantages. However, the use and applicability of cell lines are not without limitations. For example, cell lines cannot be used to reliably predict *in vivo* toxicity or to assess the toxicologic properties of new agents. Cell lines also may be ineffective in establishing mechanisms of drug metabolism or in elucidating critical tumor-host interactions. Their metabolic adaptations to *in vitro* growth may not reflect adaptations that occur *in vivo*. Nevertheless, breast cancer cell lines have been used successfully for many years to generate new hypotheses, screen new agents, and study the biology of breast cancer. Many cell lines have the advantage of being tumorigenic and thus can facilitate further studies *in vivo* in experimental animals. Provided their limitations are freely acknowledged, human breast cancer cell lines will continue to provide one of the most powerful tools in breast cancer research.

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REFERENCES

1. Thompson EW, Paik S, Brünner N, et al. Association of increased basement membrane-invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines. *J Cell Physiol* 1992;150:534.
2. Engel LW, Young NA. Human breast carcinoma cells in continuous culture: a review. *Cancer Res* 1978;38:4327.
3. Magdelenat H, Pouillart P. Steroid hormone receptors in breast cancer. In: Sheridan PJ, Blum K, Trachtenberg MC, eds. *Steroid receptors and disease: cancer autoimmune, bone and circulatory disorders*. New York: Marcel Dekker, 1988:435.
4. McGuire WL, Clark GM. The prognostic role of progesterone receptor in human breast cancer. *Semin Oncol* 1983;10:2.
5. Ravdin PM, Green S, Dorr TM, et al. Prognostic significance of progesterone receptor levels in estrogen receptor–positive patients with metastatic breast cancer treated with tamoxifen: results of a prospective Southwest Oncology Group study. *J Clin Oncol* 1992;10:1284.
6. Lippman ME, Bolan G, Huff K. The effects of estrogens and antiestrogens on hormone responsive human breast cancer cells in long term tissue culture. *Cancer Res* 1976;36:4595.
7. Lippman ME, Bolan G, Huff K. Interactions of antiestrogens with human breast cancer in long-term tissue culture. *Cancer Treat Rep* 1976;60:1421.
8. Yano T, Korkut E, Pinski J, et al. Inhibition of growth of MCF-7 MIII human breast carcinoma in nude mice by treatment with agonists or antagonists of LH-RH. *Breast Cancer Res Treat* 1992;21:35.
9. Fontana JA, Hobbs PD, Dawson MI. Inhibition of mammary carcinoma growth by retinoidal benzoic acid derivatives. *Exp Cell Biol* 1988;56:254.
10. Fontana JA. Interaction of retinoids and tamoxifen on the inhibition of human mammary carcinoma cell proliferation. *Exp Cell Biol* 1987;55:136.
11. Baumann K, Clarke R. Effects of all-*trans*-retinoic acid on proliferation and gene expression of human breast cancer cells *in vitro. Proc Am Assoc Cancer Res* 1994;35:275.
12. Soule HD, Vasquez J, Long A, Albert S, Brennan M. A human cell line from a pleural effusion derived from a human breast carcinoma. *J Natl Cancer Inst* 1973;51:1409.
13. Horwitz KB, Costlow ME, McGuire WL. MCF-7: a human breast cancer cell line with estrogen, androgen, progesterone and glucocorticoid receptors. *Steroids* 1975;26:785.
14. Clarke R, Brünner N, Katzenellenbogen BS, et al. Progression from hormone dependent to hormone independent growth in MCF-7 human breast cancer cells. *Proc Natl Acad Sci U S A* 1989;86:3649.
15. Eidne KA, Flanagan CA, Harris NS, Millar RP. Gonadotropin-releasing hormone (GnRH)-binding sites in human breast cancer cell lines and inhibitory effects of GnRH antagonists. *J Clin Endocrinol Metab* 1987;64:425.
16. Monaco ME, Lippman ME. Insulin stimulation of fatty acid synthesis in human breast cancer cells in long term tissue culture. *Endocrinology* 1977;101:1238.
17. Swisshelm K, Ryan K, Lee X, Tsou HC, Peacocke M, Sager R. Down regulation of retinoic acid receptor β in mammary carcinoma cell lines and its upregulation in senescing normal mammary epithelial cells. *Cell Growth Differ* 1994;5:133.
18. Shafie SM, Brooks SC. Effect of prolactin on growth and estrogen receptor levels of human breast cancer cells (MCF-7). *Cancer Res* 1977;37:792.
19. Huff KK, Kaufman D, Gabbay KH, Spencer EM, Lippman ME, Dickson RB. Secretion of an insulin-like growth factor-I-related protein by human breast cancer cells. *Cancer Res* 1986;46:4613.
20. Huff KK, Knabbe C, Lindsey R, et al. Multihormonal regulation of insulin-like growth factor-I-related protein in MCF-7 human breast cancer cells. *Mol Endocrinol* 1988;2:200.
21. van der Burg B, Isbrucker L, van Selm-Miltenburg AJ, de Laat SW, van Zoelen EJ. Role of estrogen-induced insulin-like growth factors in the proliferation of human breast cancer cells. *Cancer Res* 1990;50:7770.
22. Stewart A, Johnson MD, May FEB, Westley BR. Role of insulin-like growth factors and the type I insulin-like growth factor receptor in the estrogen-stimulated proliferation of human breast cancer cells. *J Biol Chem* 1990;265:21172.
23. Furlanetto RW, DiCarlo JN. Somatomedin C receptors and growth effects in human breast cancer cells maintained in long term tissue culture. *Cancer Res* 1984;44:2122.
24. Arteaga CL, Osborne CK. Growth inhibition of human breast cancer cells in vitro with an antibody aganst the Type I somatomedin receptor. *Cancer Res* 1989;49:6237.
25. Yee D, Cullen KJ, Paik S, et al. Insulin-like growth factor-II mRNA expression in human breast cancer. *Cancer Res* 1988;48:6691.
26. Clemmons DR, Camacho-Hubner C, Coronado E, Osborne CK. Insulin-like growth factor binding protein secretion by breast carcinoma cell lines: correlation with estrogen receptor status. *Endocrinology* 1990;127:2679.
27. Yee D, Favoni R, Lupu R, et al. The insulin-like growth factor binding protein BP-25 is expressed by human breast cancer cells. *Biochem Biophys Res Commun* 1989;158:38.
28. Davidson NE, Gelmann EP, Lippman ME, Dickson RB. Epidermal growth factor receptor gene expression in estrogen receptor-positive and negative human breast cancer cell lines. *Mol Endocrinol* 1987;1:216.
29. Bates SE, Davidson NE, Valverius EM, et al. Expression of transforming growth factor-α and its mRNA in human breast cancer: its regulation by estrogen and its possible functional significance. *Mol Endocrinol* 1988;2:543.
30. Clarke R, Brünner N, Katz D, et al. The effects of a constitutive production of TGF-α on the growth of MCF-7 human breast cancer cells in vitro and in vivo. *Mol Endocrinol* 1989;3:372.
31. Kern FG, Wellstein A, Flamm S, et al. Secretion of heparin binding growth factors by breast cancer cells and their role in promoting cancer cell growth. *Cancer Chemother* 1990;5:167.
32. Lehtola L, Partanen J, Sistonen L, et al. Analysis of tyrosine kinase mRNAs including four FGF receptors expressed in the MCF-7 breast cancer cells. *Int J Cancer* 1992;50:598.
33. Bronzert DA, Pantazis P, Antoniades HN, et al. Synthesis and secretion of platelet-derived growth factor by human breast cancer cell lines. *Proc Natl Acad Sci U S A* 1987;84:5763.
34. Clarke R, Dickson RB, Lippman ME. Hormonal aspects of breast cancer: growth factors, drugs and stromal interactions. *Crit Rev Oncol Hematol* 1992;12:1.
35. Clarke R, Dickson RB, Brünner N. The process of malignant progression in human breast cancer. *Ann Oncol* 1990;1:401.
36. Clarke R, Skaar T, Baumann K, et al. Hormonal carcinogenesis in breast cancer: cellular and molecular studies of malignant progression. *Breast Cancer Res Treat* 1994;31:237.
37. Keydar I, Chen L, Karby S, et al. Establishment and characterization of a cell line of human carcinoma origin. *Eur J Cancer* 1979;15:659.
38. Horwitz KB, Friedenberg GR. Growth inhibition and increase of insulin receptors in antiestrogen-resistant T47Dco human breast cancer cells by progestins: implications for endocrine therapies. *Cancer Res* 1985;45:167.
39. Cailleau R, Olive M, Cruciger QVA. Long-term human breast carcinoma cell lines of metastatic origin: preliminary characterization. *In Vitro* 1978;14:911.
40. Reddel RR, Alexander IE, Koga M, Shine J, Sutherland RL. Genetic instability and the development of steroid hormone insensitivity in cultured T 47D human breast cancer cells. *Cancer Res* 1988;48:4340.
41. Graham ML, Smith JA, Jewett PB, Horwitz KB. Heterogeneity of progesterone receptor content and remodelling by tamoxifen characterize subpopulations of cultured human breast cancer cells: analysis by quantitative dual parameter flow cytometry. *Cancer Res* 1992;52:593.
42. Sartorius CA, Groshong SD, Miller LA, et al. New T47D breast cancer cell lines for the independent study of progesterone B- and A-receptors: only antiprogestin-occupied B-receptors are switched to transcriptional agonists by cAMP. *Cancer Res* 1994;54:3868.
43. Graham ML, Dalquist KE, Horwitz KB. Simultaneous measurement of progesterone receptors and DNA indices by flow cytometry: analysis of breast cancer cell mixtures and genetic instability of the T47D line. *Cancer Res* 1989;49:3943.

44. Vignon F, Bardon S, Chalbos D, Rochefort H. Antiestrogenic effect of R5020, a synthetic progestin in human breast cancer cells in culture. *J Clin Endocrinol Metab* 1983;56:1124.
45. Horwitz KB. The antiprogestin RU38 486: receptor-mediated progestin versus antiprogestin actins screened in estrogen-insensitive T47Dco human breast cancer cells. *Endocrinology* 1985;116:2236.
46. Mockus MB, Lessey BA, Bower MA, Horwitz KB. Estrogen-insensitive progesterone receptors in a human breast cancer cell line: characterization of receptors and of a ligand exchange assay. *Endocrinology* 1982;110:1564.
47. Engel LW, Young NA, Tralka TS, Lippman ME, O'Brien SJ, Joyce MJ. Establishment and characterization of three new continuous cell lines derived from human breast carcinomas. *Cancer Res* 1978;38:3352.
48. van den Berg HW, Leahey WJ, Lynch M, Clarke R, Nelson J. Recombinant human interferon alpha increases oestrogen receptor expression in human breast cancer cells (ZR-75-1) and sensitises them to the anti-proliferative effects of tamoxifen. *Br J Cancer* 1987;55:255.
49. van den Berg HW, Lynch M, Martin J, Nelson J, Dickson GR, Crockard AD. Characterization of a tamoxifen-resistant variant of the ZR-75-1 human breast cancer cell line (ZR-75-9a1) and stability of the resistant phenotype. *Br J Cancer* 1989;59:522.
50. Kraus MH, Popescu NC, Amsbaugh SC, King CR. Overexpression of the EGF receptor-related proto-oncogene erbB-2 in human mammary tumor cell lines by different molecular mechanisms. *EMBO J* 1987;6:605.
51. Warri AM, Laine AM, Majasuo KE, Alitalo KK, Harkonen PL. Estrogen suppression of erbB2 expression is associated with increased growth rate of ZR-75-1 human breast cancer cells in vitro and in nude mice. *Int J Cancer* 1991;49:616.
52. Long B, McKibben BM, Lynch M, van den Berg HW. Changes in epidermal growth factor receptor expression and response to ligand associated with acquired tamoxifen resistance or oestrogen independence in the ZR-75-1 human breast cancer cell line. *Br J Cancer* 1992;65:865.
53. van Agthoven T, van Agthoven TL, Portengen H, Foekens JA, Dorssers LC. Ectopic expression of epidermal growth factor receptors induces hormone independence in ZR-75-1 human breast cancer cells. *Cancer Res* 1992;52:5082.
54. Poulin R, Baker D, Poirier D, Labrie F. Androgen and glucocorticoid receptor-mediated inhibition of cell proliferation by medroxyprogesterone acetate in ZR-75-1 human breast cancer cells. *Breast Cancer Res Treat* 1989;13:161.
55. Poulin R, Baker D, Poirier D, Labrie F. Multiple actions of synthetic "progestins" on the growth of ZR-75-1 human breast cancer cells: an in vitro model for the simultaneous assay of androgen, progestin, estrogen, and glucocorticoid agonistic and antagonistic activities of steroids. *Breast Cancer Res Treat* 1991;17:197.
56. Weckbecker G, Liu R, Tolcsvai L, Bruns C. Antiproliferative effects of the somatostatin analogue octreotide (SMS 201-995) on ZR-75-1 human breast cancer cells in vivo and in vitro. *Cancer Res* 1992;52:4973.
57. Theriault C, Labrie F. Multiple steroid metabolic pathways in ZR-75-1 human breast cancer cells. *J Steroid Biochem Mol Biol* 1991;38:155.
58. Poulin R, Poirier D, Merand Y, Theriault C, Belanger A, Labrie F. Extensive esterification of adrenal C19-delta 5-sex steroids to long-chain fatty acids in the ZR-75-1 human breast cancer cell line. *J Biol Chem* 1989;264:9335.
59. Roy R, Belanger A. ZR-75-1 breast cancer cells generate nonconjugated steroids from low density lipoprotein-incorporated lipoidal dehydroepiandrosterone. *Endocrinology* 1993;133:683.
60. Brünner N, Boulay V, Fojo A, Freter C, Lippman ME, Clarke R. Acquisition of hormone-independent growth in MCF-7 cells is accompanied by increased expression of estrogen-regulated genes but without detectable DNA amplifications. *Cancer Res* 1993;53:283.
61. Thompson EW, Brünner N, Torri J, et al. The invasive and metastatic properties of hormone-independent and hormone-responsive variants of MCF-7 human breast cancer cells. *Clin Exp Metastasis* 1993;11:15.
62. Clarke R, Brünner N, Thompson EW, et al. The inter-relationships between ovarian-independent growth, antiestrogen resistance and invasiveness in the malignant progression of human breast cancer. *J Endocrinol* 1989;122:331.
63. Jones DY, Schatzkin A, Green SB, et al. Dietary fat and breast cancer in the National Health and Nutrition Examination Survey I epidemiologic follow-up study. *J Natl Cancer Inst* 1987;79:465.
64. Price JE, Polyzos A, Zhang RD, Daniels LM. Tumorigenicity and metastasis of human breast carcinoma cell lines in nude mice. *Cancer Res* 1990;50:717.
65. Clarke R, Thompson EW, Leonessa F, Lippman J, McGarvey M, Brünner N. Hormone resistance, invasiveness and metastatic potential in human breast cancer. *Breast Cancer Res Treat* 1993;24:227.
66. Katzenellenbogen BS, Kendra KL, Norman MJ, Berthois Y. Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. *Cancer Res* 1987;47:4355.
67. Cho H, Ng PA, Katzenellenbogen BS. Differential regulation of gene expression by estrogen in estrogen growth-independent and -dependent MCF-7 human breast cancer cell sublines. *Mol Endocrinol* 1991; 5:1323.
68. Natoli C, Sica G, Natoli V, Serra A, Iacobelli S. Two new estrogen-supersensitive variants of the mcf-7 human breast cancer cell line. *Breast Cancer Res Treat* 1983;3:23.
69. McLeskey SW, Kurebayashi J, Honig SF, et al. Fibroblast growth factor 4 transfection of MCF-7 cells produces cell lines that are tumorigenic and metastatic in ovariectomized or tamoxifen-treated athymic nude mice. *Cancer Res* 1993;53:2168.
70. Kurebayashi J, McLeskey SW, Johnson MD, Lippman ME, Dickson RB, Kern FG. Quantitative demonstration of spontaneous metastasis by MCF-7 human breast cancer cells cotransfected with fibroblast growth factor 4 and LacZ. *Cancer Res* 1993;53:2178.
71. Lasfargues EY, Ozzello L. Cultivation of human breast carcinomas. *J Natl Cancer Inst* 1958;21:1131.
72. Horwitz KB, Zava DT, Thilagar AK, Jensen EM, McGuire WL. Steroid receptor analyses of nine human breast cancer cell lines. *Cancer Res* 1978;38:2434.
73. Hall RE, Lee CSL, Alexander IE, Shine J, Clark CL, Sutherland RL. Steroid hormone receptor gene expression in human breast cancer cells: inverse relationship between oestrogen and glucocorticoid receptor messenger RNA levels. *Int J Cancer* 1990;46:1081.
74. Castles CG, Fuqua SA, Klotz DM, Hill SM. Expression of a constitutively active estrogen receptor variant in the estrogen receptor-negative BT-20 human breast cancer cell line. *Cancer Res* 1993;53:5934.
75. Fuqua SAW, Fitzgerald SD, Chamness GC, et al. Variant human breast tumor estrogen receptor with constitutive transcriptional activity. *Cancer Res* 1991;51:105.
76. Kurokawa M, Michelangeli VP, Findlay DM. Induction of calcitonin receptor expression by glucocorticoids in T47D human breast cancer cells. *J Endocrinol* 1991;130:321.
77. Ozzello L, Sordat B, Merenda C, Carrel S, Hurlimann J, Mach JP. Transplantation of a human mammary carcinoma cell line (BT 20) into nude mice. *J Natl Cancer Inst* 1974;52:1669.
78. Lasfargues EY, Coutinho WG, Redfield ES. Isolation of two human tumor epithelial cell lines from solid breast carcinomas. *J Natl Cancer Inst* 1978;61:967.
79. Horwitz KB, Mockus MB, Lessey BA. Variant T47D human breast cancer cells with high progesterone-receptor levels despite estrogen and antiestrogen resistance. *Cell* 1982;28:633.
80. Ballare C, Bravo AI, Laucella S, et al. DNA synthesis in estrogen receptor positive human breast cancer takes place preferentially in estrogen receptor-negative cells. *Cancer* 1989;64:842.
81. Shek LL, Godlophin W. Survival with breast cancer: the importance of estrogen receptor quantity. *Eur J Cancer Clin Oncol* 1989;25:243.
82. Clark GM, McGuire WL. Steroid receptors and other prognostic factors in primary breast cancer. *Semin Oncol* 1988;15:20.
83. Skoog L, Humla S, Axelsson M, et al. Estrogen receptor levels and survival of breast cancer patients. *Acta Oncol* 1987;26:95.
84. Halter SA, Fraker LD, Adcock D, Vick S. Effect of retinoids on xenotransplanted human mammary carcinoma cells in athymic mice. *Cancer Res* 1988;48:3733.
85. Cailleau R, Young R, Olive M, Reeves WJ. Breast tumor cell lines from pleural effusions. *J Natl Cancer Inst* 1974;53:661.
86. Leonessa F, Green D, Licht T, et al. MDA435/LCC6 and MDA435/LCC6MDRI: ascites models of human breast cancer. *Br J Cancer* 1996;73:154.
87. Osborne CK, Coronado E, Allred DC, Wiebe V, DeGregorio M. Acquired tamoxifen resistance: correlation with reduced breast tumor levels of tamoxifen and isomerization of trans-4-hydroxytamoxifen. *J Natl Cancer Inst* 1991;83:1477.
88. Meschter CL, Connolly JM, Rose DP. Influence of regional location of the inoculation site and dietary fat on the pathology of MDA-MB-435 human breast cancer cell-derived tumors growing in nude mice. *Clin Exp Metastasis* 1992;10:167.

89. Rose DP, Connolly JM, Meschter CL. Effect of dietary fat on human breast cancer growth and lung metastasis in nude mice. *J Natl Cancer Inst* 1991;83:1491.
90. Rose DP, Hatala MA, Connolly JM, Rayburn J. Effect of diets containing different levels of linoleic acid on human breast cancer growth and lung metastasis in nude mice. *Cancer Res* 1993;53:4686.
91. Rose DP, Connolly JM. Influence of dietary fat intake on local recurrence and progression of metastases arising from MDA-MB-435 human breast cancer cells in nude mice after excision of the primary tumor. *Nutr Cancer* 1992;18:113.
92. Brünner N, Thompson EW, Spang-Thomsen M, Rygaard J, Dano K, Zwiebel JA. LacZ transduced human breast cancer xenografts as an in vivo model for the study of invasion and metastasis. *Eur J Cancer* 1992;28A:1989.
93. Siciliano MJ, Barker PE, Cailleau R. Mutually exclusive genetic signatures of human breast tumor cell lines with a common chromosomal marker. *Cancer Res* 1979;39:919.
94. Osborne CK, Lippman ME. Human breast cancer in tissue culture. In: McGuire WL, ed. *Breast cancer advances in research and treatment.* New York: Plenum, 1978:103.
95. Kaplan O, Jaroszewski JW, Faustino PJ, et al. Toxicity and effects of epidermal growth factor on the glucose metabolism of MDA-468 human breast cancer cells. *J Biol Chem* 1990;265:13641.
96. McLeskey SW, Ding IY, Lippman ME, Kern FG. MDA-MB-134 breast carcinoma cells overexpress fibroblast growth factor (FGF) receptors and are growth-inhibited by FGF ligands. *Cancer Res* 1994;54:523.
97. Langton BC, Crenshaw MC, Chao LA, Stuart SG, Akita RW, Jackson JE. An antigen immunologically related to the external domain of gp185 is shed from nude mouse tumors overexpressing the c-erbB2 (HER-2/neu) oncogene. *Cancer Res* 1991;51:2593.
98. Kennedy DG, Clarke R, van den Berg HW, Murphy RF. The kinetics of methotrexate polyglutamate formation and efflux in a human breast cancer cell line (MDA-MB-436): the effect of insulin. *Biochem Pharmacol* 1983;32:41.
99. Kennedy DG, van den Berg HW, Clarke R, Murphy RF. The effect of the rate of cell proliferation on the synthesis of methotrexate poly-τ-glutamates in two human breast cancer cell lines. *Biochem Pharmacol* 1985;34:3087.
100. Clarke R, van den Berg HW, Kennedy DG, Murphy RF. Reduction of the antimetabolic and antiproliferative effects of methotrexate by 17β-estradiol in a human breast carcimoma cell line (MDA-MB-436). *Eur J Cancer Clin Oncol* 1983;19:19.
101. Clarke R, van den Berg HW, Kennedy DG, Murphy RF. Oestrogen receptor status and the response of human breast cancer cells to a combination of methotrexate and 17β-estradiol. *Br J Cancer* 1985;51:365.
102. Clarke R, van den Berg HW, Murphy RF. Tamoxifen and 17β-estradiol reduce the membrane fluidity of human breast cancer cells. *J Natl Cancer Inst* 1990;82:1702.
103. Fogh J. Cell lines established from human tumors. In: Fogh J, ed. *Human tumor cell lines in vitro.* New York: Plenum, 1975:115.
104. Alpet O, Yamaguchi K, Hitomi J, Honda S, Matsushima T, Abe K. The presence of c-erbB-2 gene product-related protein in culture medium conditioned by breast cancer cell line SK-BR-3. *Cell Growth Differ* 1990;1:591.
105. Goldman R, Levy MB, Peles E, Yarden Y. Heterodimerization of the erbB-1 and erbB-2 receptors in human breast carcinoma cells: a mechanism for receptor transregulation. *Biochemistry* 1990;29:11024.
106. Volpi A, Savini S, Zoli W, et al. An efficient method for culturing human breast epithelium: analysis of results. *Tumori* 1991;77:460.
107. Zoli W, Volpi A, Bonaguri C, et al. An efficient method for culturing human breast carcinoma to evaluate antiblastic drug activity in vitro: experience on 136 primary cancers and 116 recurrences. *Breast Cancer Res Treat* 1990;17:231.
108. Dairkee SH, Deng G, Stampfer MR, Waldman FM, Smith HS. Selective cell culture of primary breast carcinoma. *Cancer Res* 1995; 55:2516.
109. McCallum HM, Lowther GW. Long-term culture of primary breast cancer in defined medium. *Breast Cancer Res Treat* 1996;39:247.
110. Jiang S-Y, Jordan VC. Growth regulation of estrogen receptor negative breast cancer cells transfected with estrogen receptor cDNAs. *J Natl Cancer Inst* 1992;84:580.
111. Pilat MJ, Christman JK, Brooks SC. Characterization of the estrogen receptor transfected MCF10A breast line 139B6. *Breast Cancer Res Treat* 1996;37:253.
112. Pasqualini JR, Chetrite G, Nestour EL. Control and expression of oestrone sulphatase activities in human breast cancer. *J Endocrinol* 1996;150:S99.
113. Hulka BS, Stark AT. Breast cancer: cause and prevention. *Lancet* 1995;346:883.
114. Brunner N, Zugmaier G, Bano M, et al. Endocrine therapy of human breast cancer cells: the role of secreted polypeptide growth factors. *Cancer Cells* 1989;1:81.
115. Bei M, Foegh M, Ramwell PR, Clarke R. Specific high affinity binding sites for 17β-estradiol in rat heart smooth muscle cells. *J Steroid Biochem Mol Biol* 1996;58:83.
116. Lavigne MC, Ramwell PW, Clarke R. Growth and phenotypic characterization of porcine coronary artery smooth muscle cells. *In Vitro Cell Dev Biol* 1999;35:136.
117. Lavigne MC, Ramwell PW, Clarke R. Inhibition of estrogen receptor function promotes porcine coronary artery smooth muscle cell proliferation. *Steroids* 1999;64:472.
118. Amadori D, Bertoni L, Flamigni A, et al. Establishment and characterization of a new cell line from primary human breast carcinoma. *Breast Cancer Res Treat* 1993;28:251.
119. Petersen OW, van Deurs B, Nielsen KV, et al. Differential tumorigenicity of two autologous human breast carcinoma cell lines, HMT-3939S1 and HMT-3939S8, established in serum-free medium. *Cancer Res* 1990;50:1257.
120. Kurebayashi J, Kurosumi M, Sonoo H. A new human breast cancer cell line, KPL-3C, secretes parathyroid hormone-related protein and produces tumors associated with microcalcifications in nude mice. *Br J Cancer* 1996;74:200.
121. Thompson EW, Sung V, Lavigne M, et al. LCC15-MB: a human breast cancer cell line from a femoral bone metastasis. *Clin Exp Metastasis* 1999;17:193.
122. Sung V, Gilles C, Murray A, et al. The LCC15-MB human breast cancer cell line expresses osteopontin and exhibits an invasive and metastatic phenotype. *Exp Cell Res* 1998;241:273.
123. Hackenberg R, Lüttchens S, Hofmann J, Kunzmann R, Holzel F, Schulz K-D. Androgen sensitivity of the new breast cancer cell line MFM-233. *Cancer Res* 1991;51:5722.
124. Bronzert DA, Greene GL, Lippman ME. Selection and characterization of a breast cancer cell line resistant to the antiestrogen LY 117018. *Endocrinology* 1985;117:1409.
125. Nawata H, Chang MJ, Bronzert D, Lippman ME. Estradiol independent growth of a subline of MCF-7 human breast cancer cells in culture. *J Biol Chem* 1981;256:6895.
126. Nawata H, Bronzert D, Lippman ME. Isolation and characterization of a tamoxifen resistant cell line derived from MCF-7 human breast cancer cells. *J Biol Chem* 1981;256:5016.
127. van den Berg HW, Clarke R. Preliminary characterization of a tamoxifen resistant variant of the oestrogen responsive human breast cancer cell line ZR-75-1. *Br J Cancer* 1985;52:421.
128. Gottardis MM, Jordan VC. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res* 1988;48:5183.
129. Osborne CK, Coronado EB, Robinson JP. Human breast cancer in athymic nude mice: cytostatic effects of long-term antiestrogen therapy. *Eur J Cancer Clin Oncol* 1987;23:1189.
130. Mullick A, Chambon P. Characterization of the estrogen receptor in two antiestrogen-resistant cell lines, LY2 and T47D. *Cancer Res* 1990;50:333.
131. Seibert K, Shafie SM, Triche TJ, et al. Clonal variation of MCF-7 breast cancer cells in vitro and in athymic nude mice. *Cancer Res* 1983;43:2223.
132. van Steenbrugge GJ, Groen M, van Kreuningen A, De Jong FH, Gallee MWP, Schroeder FH. Transplantable human prostatic carcinoma (PC-82) in athymic nude mice (III). Effects of estrogens on the growth of the tumor. *Prostate* 1988;12:157.
133. Brünner N, Svenstrup B, Spang-Thompsen M, Bennet P, Nielsen A, Nielsen JJ. Serum steroid levels in intact and endocrine ablated Balb\c nude mice and their intact litter mates. *J Steroid Biochem* 1986;25:429.
134. Clarke R, Lippman ME. Antiestrogen resistance: mechanisms and reversal. In: Teicher BA, ed. *Drug resistance in oncology.* New York: Marcel Dekker, 1992:501.
135. Jiang SY, Parker CJ, Jordan VC. A model to describe how a point mutation of the estrogen receptor alters the structure-function relationship of antiestrogens. *Breast Cancer Res Treat* 1993;26:139.

136. Jiang SY, Langan-Fahey SM, Stella AL, McCague R, Jordan VC. Point mutation of estrogen receptor (ER) in the ligand-binding domain changes the pharmacology of antiestrogens in ER-negative breast cancer cells stably expressing complementary DNAs for ER. *Mol Endocrinol* 1992;6:2167.
137. Engelsman E. Therapy of advanced breast cancer; a review. *Eur J Cancer Clin Oncol* 1983;19:1775.
138. Gockerman JP, Spremulli EN, Raney M, Logan T. Randomized comparison of tamoxifen versus diethylstilbestrol in estrogen receptor-positive or -unknown metastatic breast cancer: a southeastern cancer study group trial. *Cancer Treat Rep* 1986;70:1199.
139. Vogel CL, East DR, Voigt W, Thomsen S. Response to tamoxifen in estrogen receptor-poor metastatic breast cancer. *Cancer* 1987; 60:1184.
140. Howell A, Dodwell DJ, Anderson H, Redford J. Response after withdrawal of tamoxifen and progestogens in advanced breast cancer [See comments]. *Ann Oncol* 1992;3:611.
141. Belani CP, Pearl P, Whitley NO, Aisner J. Tamoxifen withdrawal response. Report of a case. *Arch Intern Med* 1989;149:449.
142. Stein W, Hortobagyi GN, Blumenschein GR. Response of metastatic breast cancer to tamoxifen withdrawal: report of a case. *J Surg Oncol* 1983;22:45.
143. McIntosh IH, Thynne GS. Tumour stimulation by anti-oestrogens. *Br J Surg* 1977;64:900.
144. Canney PA, Griffiths T, Latief TN, Priestman TJ. Clinical significance of tamoxifen withdrawal response. *Lancet* 1989;1:36.
145. Beex LVAM, Pieters GFFM, Smals AGH, Koenders AJM, Benraad TJ, Kloppenborg PWC. Diethylstilbestrol versus tamoxifen in advanced breast cancer. *N Engl J Med* 1981;304:1041.
146. Brünner N, Frandsen TL, Holst-Hansen C, et al. MCF7/LCC2: A 4-hydroxytamoxifen resistant human breast cancer variant which retains sensitivity to the steroidal antiestrogen ICI 182,780. *Cancer Res* 1993;53:3229.
147. Coopman P, Garcia M, Brünner N, Derocq D, Clarke R, Rochefort H. Antiproliferative and antiestrogenic effects of ICI 164,384 in 4-OH-tamoxifen-resistant human breast cancer cells. *Int J Cancer* 1994;56:295.
148. Nicholson RI, Gee JMW, Anderson E, et al. Phase I study of a new pure antiestrogen ICI 182,780 in women with primary breast cancer: immunohistochemical analysis. *Breast Cancer Res Treat* 1993;27:135.
149. Brünner N, Boysen B, Jirus S, et al. MCF7/LCC9: An antiestrogen-resistant MCF-7 variant in which acquired resistance to the steroidal antiestrogen ICI 182,780 confers an early cross-resistance to the non-steroidal antiestrogen tamoxifen. *Cancer Res* 1997;57:3486.
150. Schneider J, Bak M, Efferth T, Kaufmann M, Mattern J, Volm M. P-glycoprotein expression in treated and untreated human breast cancer. *Br J Cancer* 1989;60:815.
151. Trock BJ, Leonesa F, Clarke R. Multidrug resistance in breast cancer: a meta analysis of MDR1/gp170 expression and its possible functional significance. *J Natl Cancer Inst* 1997;89:917.
152. Sanfilippo O, Ronchi E, De Marco C, Di Fronzo G, Silvestrini R. Expression of P-glycoprotein in breast cancer tissue and in vitro resistance to doxorubicin and vincristine. *Eur J Cancer* 1991;27:155.
153. Salmon SE, Grogan TM, Miller T, Scheper R, Dalton WS. Prediction of doxorubicin resistance in vitro in myeloma, lymphoma and breast cancer by P-glycoprotein staining. *J Natl Cancer Inst* 1989;81:696.
154. Veneroni S, Zaffaroni N, Daidone MG, Benini E, Villa R, Silvestrini R. Expression of P-glycoprotein and in vitro or in vivo resistance to doxorubicin and cisplatin in breast and ovarian cancers. *Eur J Cancer* 1994;30A:1002.
155. Vickers PJ, Dickson RB, Shoemaker R, Cowan KH. A multidrug-resistant MCF-7 human breast cancer cell line which exhibits cross-resistance to antiestrogens and hormone independent tumor growth. *Mol Endocrinol* 1988;2:886.
156. Willingham MC, Cornwell MM, Cardarelli CO, Gottesman MM, Pastan I. Single cell analysis of daunomycin uptake and efflux in multidrug resistant and sensitive KB cells: effects of verapamil and other drugs. *Cancer Res* 1986;46:5941.
157. Scudiero DA, Monks A, Sausville EA. Cell line designation change: multidrug-resistant cell line in the NCI anticancer screen. *J Natl Cancer Inst* 1998;90:862.
158. Zyad A, Bernard J, Clarke R, Tursz T, Brockhaus M, Chouaib S. Human breast cancer cross-resistance to TNF and adriamycin: relationship to MDR1, MnSOD and TNF gene expression. *Cancer Res* 1994;54:825.
159. Doroshow JH, Akman S, Esworthy S, Chu FF, Burke T. Doxorubicin resistance is conferred by selective enhancement of intracellular glutathione peroxidase or superoxide dismutase content in human MCF-7 breast cancer cells. *Free Radic Res Commun* 1991;12–13, part 2:779.
160. Iwamoto S, Takeda K. Possible cytotoxic mechanisms of TNF in vitro. *Hum Cell* 1990;3:107.
161. Batist G, Tuple A, Sinha BK, Katki AG, Myers CE, Cowan KH. Overexpression of a novel anionic glutathione transferase in multidrug-resistant human breast cancer cells. *J Biol Chem* 1986;261:15544.
162. Sinha BK, Mimnaugh EG, Rajagopalan S, Myers CE. Adriamycin activation and oxygen free radical formation in human breast tumor cells: protective role of glutathione peroxidase in adriamycin resistance. *Cancer Res* 1989;49:3844.
163. Ford JM, Bruggemann EP, Pastan I, Gottesman MM, Hait WN. Cellular and biochemical characterization of thioxanthenes for reversal of multidrug resistance in human and murine cell lines. *Cancer Res* 1990;50:1748.
164. Clarke R, Currier S, Kaplan O, et al. Effect of P-glycoprotein expression on sensitivity to hormones in MCF-7 human breast cancer cells. *J Natl Cancer Inst* 1992;84:1506.
165. Kaplan O, Jaroszewski JW, Clarke R, et al. The multidrug resistance phenotype: 31P NMR characterization and 2-deoxyglucose toxicity. *Cancer Res* 1991;51:1638.
166. Leonessa F, Green D, Licht T, et al. MDA435/LCC6 and MDA435/LCC6^{MDR1}: ascites models of human breast cancer. *Br J Cancer* 1996;73:154.
167. Ip MM, Darcy KM. Three-dimensional mammary culture model systems. *J Mammary Gland Biol Neoplasia* 1996;1:91.
168. Petersen OW, Ronnov L, Bissell MJ. The microenvironment of the breast: three-dimensional models to study the roles of the stroma and the extracellular matrix in function and dysfunction. *Breast J* 1995;1:22.
169. Baibakov BA, Chipisheva TA, Guelsheva VI, et al. Organotypic growth and differentiation of human mammary gland in sponge-gel matrix supported histoculture. *In Vitro Cell Dev Biol* 1994;30A:490.
170. Dairkee SH, Heid HW. Cytokeratin profile of immunomagnetically separated epithelial subsets of the mammary gland. *In Vitro Cell Dev Biol* 1993;29A:427.
171. Speirs V, Green AR, Walton DS, et al. Short-term primary culture of epithelial cells derived from human breast tumours. *Br J Cancer* 1998;78:1421.
172. Stampfer M, Hallowes RC, Hackett AJ. Growth of normal human mammary cells in culture. *In Vitro* 1980;16:415.
173. Stampfer MR, Bartley JC. Human mammary epithelial cells in culture: differentiation and transformation. In: Lippman ME, Dickson RB, eds. *Breast cancer: cellular and molecular biology*. Boston: Kluwer Academic, 1988:1.
174. Smith HS. In vitro models in human breast cancer. In: Harris J, Hellman S, Henderson IC, Kinne D, eds. *Breast diseases*. New York: Lippincott–Raven, 1994.
175. Soule HD, Maloney TM, Wolman SR, et al. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer Res* 1990;50:6075.
176. Lundholt BK, Madsen MW, Lykkesfeldt AE, Petersen OW, Briand P. Characterization of a nontumorigenic human breast epithelial cell line stably transfected with the human estrogen receptor (ER) cDNA. *Mol Cell Endocrinol* 1996;119:47.
177. Clark R, Stampfer MR, Milley R, et al. Transformation of human mammary epithelial cells with oncogenic viruses. *Cancer Res* 1988;48:4689.
178. Valverius EM, Ciardiello F, Heldin NE, et al. Stromal influences on transformation of human mammary epithelial cells overexpressing c-myc and sv40t. *J Cell Physiol* 1990;145:207.
179. Pierce JH, Arnstein P, DiMarco E, et al. Oncogenic potential of erbb-2 in human mammary epithelial cells. *Oncogene* 1991;6:1189.
180. Tait L, Soule H, Russo J. Ultrastructural and immunocytochemical characterization of an immortalized human breast epithelial cell line, MCF-10. *Cancer Res* 1990;50:6087.
181. Ciardello F, Gottardis M, Basolo F, et al. Additive effects of c-erbB-2, c-Ha-ras, and transforming growth factor-α genes on in vitro transformation of human mammary epithelial cells. *Mol Carcinog* 1992; 6:43.
182. Ochieng J, Basolo F, Albini A, et al. Increased invasive, chemotactic and locomotive abilities of c-Ha-ras-transformed human breast epithelial cells. *Invasion Metastasis* 1991;11:38.
183. Miller FR, Soule HD, Tait L, et al. Xenograft model of progressive human proliferative disease. *J Natl Cancer Inst* 1993;85:1725.

184. Dawson PJ, Wolman SR, Tait L, Heppner GH, Miller FR. MCF10-AT: a model for the evolution of cancer from proliferative breast disease. *Am J Pathol* 1996;148:313.
185. Iravani S, Mora L, Miller FR, Dawson PJ. Altered expression of c-*erbb*B-2, DF3, B72.3, p53 and Ki-67 with progression and differentiation to two distinct histologic types of invasive carcinoma in the MCF10AT human xenograft model of proliferative breast disease. *Int J Oncol* 1998;12:369.
186. Gaffney EV. A cell line (HBL-100) established from human breast milk. *Cell Tissue Res* 1982;227(3):563.
187. Krief P, Saint-Ruf C, Bracke M, et al. Acquisition of tumorigenic potential in the human myoepithelial HBL100 cell line is associated with decreased expression of HLA class I, class II and integrin beta 3 and increased expression of c-myc. *Int J Cancer* 1989;43:658.
188. Caron de Fromentel C, Nardeux PC, Soussi T, et al. Epithelial HBL-100 cell line derived from milk of an apparently healthy woman harbours SV40 genetic information. *Exp Cell Res* 1985;160:83.
189. Decloitre F, Cassingena R, Estrade S, Martin M. Concomitant alterations of microfilaments and microtubules in human epithelial cells (HBL-100) in relation to their malignant conversion. *Tumour Biol* 1991;12:111.
190. Marlhens F, Saint-Ruf C, Nardeux P, et al. Karyotype evolution of the human HBL-100 cell line and mapping of the integration site of SV40 DNA. *Ann Genet* 1988;31:81.
191. Saint-Ruf C, Nardeux P, Estrade S, et al. Accelerated malignant conversion of human HBL-100 cells by the v-Ki-ras oncogene. *Exp Cell Res* 1988;176:60.
192. Dhaliwal MK, Giovanella BC, Pathak S. Cytogenetic characterization of two human milk-derived cell line (HBL-100) passages differing in tumorigenicity. *Anticancer Res* 1990;10:113.
193. Lebeau J, Le Chalony C, Prosperi MT, Goubin G. Constitutive overexpression of a 89 kDa heat shock protein gene in the HBL100 human mammary cell line converted to a tumorigenic phenotype by the EJ/T24 Harvey-ras oncogene. *Oncogene* 1991;6:1125.
194. Robert-Guroff M, Stern TL, Richardson ES, Giovanella BC, Michaels FH. Presence of Mason-Pfizer monkey virus in some stocks of the human HBL-100 mammary epithelial cell line. *J Natl Cancer Inst* 1996;88:372.
195. Vanhamme L, Szpirer C. Transforming activity of the human mammary line HBL100 DNA is associated with SV40 large T antigen genetic information integrated in its genome. *Carcinogenesis* 1988;9:653.
196. Saint-Ruf C, Nardeux P, Cebrian J, Lacasa M, Lavialle C, Cassingena R. Molecular cloning and characterization of endogenous SV40 DNA from human HBL-100 cells. *Int J Cancer* 1989;44:367.
197. Rao KV, Williams RE, Fox CF. Altered glucocorticoid binding and action in response to epidermal growth factor in HBL100 cells. *Cancer Res* 1987;47:5888.
198. Wellner RB, He XJ, Marmary Y, Baum BJ. Functional beta-adrenergic receptors in a human mammary cell line (HBL-100). *Biochem Pharmacol* 1988;37:3035.
199. Peyrat JP, Bonneterre J. Type I IGF receptor in human breast diseases. *Breast Cancer Res Treat* 1992;22:59.
200. Laherty RF, Balcavage WX, Goff C, et al. HBL-100 cells do not secrete casein and lack prolactin and estradiol receptors. *In Vitro Cell Dev Biol* 1990;26:933.
201. Bagheri-Yarmand R, Liu JF, Ledoux D, Morere JF, Crepin M. Inhibition of human breast epithelial HBL100 cell proliferation by a dextran derivative (CMDB7): interference with the FGF2 autocrine loop. *Biochem Biophys Res Commun* 1997;239:424.
202. Schirrmacher V. Cancer metastasis: experimental approaches, theroetical concepts and impacts for treatment strategies. *Adv Cancer Res* 1985;43:1.
203. Terranova VP, Hujanen ES, Martin GR. Basement membrane and the invasive activity of metastatic tumor cells. *J Natl Cancer Inst* 1986; 77:311.
204. Barsky SH, Siegal GP, Jannotta F, Liotta LA. Loss of basement membrane components by invasive tumors but not by their benign counterparts. *Lab Invest* 1994;49:140.
205. Liotta LA, Rao CN, Barsky SH. Tumor invasion and the extracellular matrix. *Lab Invest* 1994;49:636.
206. Barsky SH, Togo S, Garbisa S, Liotta LA. Type IV collagenase immunoreactivity in invasive breast carcinoma. *Lancet* 1994;1:296.
207. Kleinman HK, McGarvey ML, Hassell JR, et al. Basement membrane complexes with biological activity. *Biochemistry* 1986;25:312.
208. Bae S-N, Arand G, Azzam H, et al. Molecular and cellular analysis of basement membrane invasion by human breast cancer cells in Matrigel-based in vitro assays. *Breast Cancer Res Treat* 1993;24:241.
209. Albini A, Iwamoto Y, Kleinman HK, et al. A rapid in vitro assay for quantitating the invasive potential of tumor cells. *Cancer Res* 1987;47:3239.
210. Frandsen TL, Boysen BE, Jirus S, et al. Experimental models for the study of human cancer cell invasion and metastasis. *Fibrinolysis* 1992;6[Suppl 4]:71.
211. Noel CT, Reed MJ, Jacobs HS, James VHT. The plasma concentration of oestrone sulphate in postmenopausal women: lack of diurnal variation, effect of ovariectomy, age and weight. *J Steroid Biochem* 1981;14:1101.
212. Thompson EW, Katz D, Shima TB, Wakeling AE, Lippman ME, Dickson RB. ICI 164,384: a pure antagonist of estrogen-stimulated MCF-7 cell proliferation and invasiveness. *Cancer Res* 1989;49:6929.
213. Thompson EW, Torri J, Sabol M, et al. Oncogene-induced basement membrane invasiveness in human mammary epithelial cells. *Clin Exp Metastasis* 1994;12:181.
214. Shi YE, Torri J, Yieh L, et al. Expression of 67 kDa laminin receptor in human breast cancer cells: regulation by progestins. *Clin Exp Metastasis* 1993;11:251.
215. Cattoretti G, Andreola S, Clemente C, D'Amato L, Rilke F. Vimentin and P53 expression in epidermal growth factor receptor-positive oestrogen receptor-negative breast carcinomas. *Br J Cancer* 1988;57:353.
216. Raymond WA, Leong AS-Y. Co-expression of cytokeratin and vimentin intermediate filament proteins in benign and neoplastic breast epithelium. *J Pathol* 1989;157:299.
217. Raymond WA, Leong AS-Y. A new prognostic parameter in breast carcinoma? *J Pathol* 1989;158:107.
218. Domagala W, Lasota J, Bartowiak J, Weber K, Osborne M. Vimentin is preferentially expressed in human breast carcinomas with low estrogen receptor and high Ki67 growth fraction. *Am J Pathol* 1994;136:219.
219. Domagala W, Leszek W, Lasota J, Weber K, Osborne M. Vimentin is preferentially expressed in high grade ductal and medullary, but not in lobular breast carcinomas. *Am J Pathol* 1994;137:1059.
220. Gamallo C, Palacios J, Suarez A, et al. Correlation of E-cadherin expression with differentiation grade and histological type in breast carcinoma. *Am J Pathol* 1993;142:987.
221. Rasbridge SA, Gillett CE, Sampson SA, Walsh FS, Millis RR. Epithelial (E-) and placental (P-) cadherin cell adhesion molecule expression in breast carcinoma. *J Pathol* 1993;169:245.
222. Oka H, Shiozaki H, Kobayashi K, et al. Expression of E-cadherin cell adhesion molecule in human breast cancer tissues and its relationship to metastases. *Cancer Res* 1993;53:1696.
223. Thiery J-P, Boyer B, Tucker G, Gavrilovic J, Valles AM. Adhesion mechanisms in embryogenesis and in cancer invasion and metastasis. *Ciba Found Symp* 1988;141:48.
224. Sommers SL, Byers SW, Thompson EW, Torri JA, Gelmann EP. Differentiation state and invasiveness of human breast cancer cell lines. *Breast Cancer Res Treat* 1994;325.
225. Sommers CL, Heckford SE, Skerker JM, et al. Loss of epithelial markers and acquisition of vimentin expression in adriamycin- and vinblastine resistant human breast cancer cells. *Cancer Res* 1992;52:5190.
226. Brünner N, Johnson MD, Holst-Hansen C, Kiilgaard JF, Thompson EW, Clarke R. Acquisition of estrogen independence and antiestrogen resistance in breast cancer: association with the invasive and metastatic phenotype. *Endocr Related Cancer* 1995;2:27.
227. Ura H, Bonfil RD, Reich R, Reddel RR, Pfeifer A, Harris CC. Expression of type IV collagenase and procollagen genes and its correlation with the tumorigenic, invasive and metastatic abilities of oncogene-transformed human bronchial epithelial cells. *Cancer Res* 1989;49:4615.
228. Bonfil DR, Reddel RR, Ura H, et al. Invasive and metastatic potential of a v-Ha-ras transformed human bronchial epithelial cell line. *J Natl Cancer Inst* 1989;81:587.
229. Thorgeirsson UP, Turpeenniemi-Hujanen T, Williams JE, et al. NIH/3T3 cells transfected with human tumor cDNA containing activated ras oncogenes express the metastatic phenotype in nude mice. *Mol Cell Biol* 1985;5:259.
230. Albini A, Graf J, Kitten GT, et al. 17β-estradiol regulates and V-Ha-ras transfection constitutively enhances MCF-7 breast cancer cell interactions with basement membrane. *Proc Natl Acad Sci U S A* 1986;83:8182.
231. Sommers CL, Papageorge A, Wilding G, Gelmann EP. Growth properties and tumorigenesis of MCF-7 cells transfected with isogenic mutants of rasH. *Cancer Res* 1990;50:67.
232. Van Roy F, Mareel M, Vleminckx K, et al. Hormone sensitivity in vitro and in vivo of v-ras-transfected MCF-7 cell derivatives. *Int J Cancer* 1990;46:522.

233. Muschel RJ, Williams JE, Lowy DR, Liotta LA. Harvey ras induction of metastatic potential depends upon oncogene activation and type of recipient cell. *Am J Pathol* 1985;121:1.
234. Nicholson GL, Gallick GE, Sphon WH, Lembo TM, Tainsky MA. Transfection of activated c-Ha-rasEJ/pSV2neo genes into rat mammary cells: rapid stimulation of clonal diversification of spontaneous metastatic and cell surface properties. *Oncogene* 1992;7:1127.
235. De Vita VT. Principles of chemotherapy. In: De Vita VT, Hellman S, Rosenberg SA, eds. *Cancer principles and practice of oncology,* 3rd ed. Philadelphia: JB Lippincott, 1989:276.
236. Fracchia AA, Knapper WH, Carey JT, Farrow JH. Intrapleural chemotherapy for effusion from metastatic breast cancer. *Cancer* 1970; 26:626.
237. Van Netten JP, Armstrong JB, Carlyle SS, et al. Estrogen receptor distribution in the peripheral, intermediate and central regions of breast cancers. *Eur J Cancer Clin Oncol* 1988;24:1885.
238. Meltzer P, Leibovitz A, Dalton W, et al. Establishment of two new cell lines derived from human breast carcinomas with HER2/neu amplification. *Br J Cancer* 1991;63:727.
239. Whitescarver J. Problems of in vitro culture of human mammary tumor cells. *J Invest Dermatol* 1974;63:58.
240. Foley JF, Aftonomos BT. Growth of human breast neoplasms in cell culture. *J Natl Cancer Inst* 1965;34:217.
241. Bastert G, Fortmeyer HP, Eichholz H, Michel RT, Huck R, Schmidt-Matthiesen H. Human breast cancer in thymus aplastic nude mice. In: Bastert GB, ed. *Thymus-aplastic nude mice and rats in clinical oncology.* New York: Verlag, 1981:157.
242. Berger DP, Winterhalter BR, Fiebig HH. Establishment and characterization of human tumor xenografts in thymus-aplastic nude mice. In: Fiebig HH, Berger DP, eds. *Immunodeficient mice in oncology.* Basel: Karger, 1992:23.
243. Federoff S, Evans VJ, Perry VP, Vincent MM, eds. *Manual of the Tissue Culture Association.* 1975:53.
244. Gibson SL, Hilf R. Regulation of estrogen-binding capacity by insulin in 7,12-dimethylbenz(*a*)anthracene-induced mammary tumors in rats. *Cancer Res* 1980;40:2343.
245. Barnes D, Sato G. Growth of a human mammary tumor cell line in a serum free medium. *Nature* 1978;281:388.
246. Vignon F, Bouton MM, Rochefort H. Antiestrogens inhibit the mitogenic effect of growth factors on breast cancer cells in the total absence of estrogens. *Biochem Biophys Res Commun* 1987; 146:1502.
247. Koga M, Sutherland RL. Epidermal growth factor partially reverses the inhibitory effects of antiestrogens on T47D human breast cancer cell growth. *Biochem Biophys Res Commun* 1987;146:738.
248. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. Phenol red in tissue culture is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *Proc Natl Acad Sci U S A* 1986;83:2496.
249. Bindal RD, Carlson KE, Katzenellenbogen BS, Katzenellenbogen JA. Lipophylic impurities, not phenolsulfonphthalein, account for the estrogenic properties in commercial preparations of phenol red. *J Steroid Biochem* 1988;31:287.
250. Darbre P, Yates J, Curtis S, King RJB. Effect of estradiol on human breast cancer cells in culture. *Cancer Res* 1983;43:349.
251. Vignon F, Terqui M, Westley B, Derocq D, Rochefort H. Effects of plasma estrogen sulfates in mammary cancer cells. *Endocrinology* 1980;106:1079.
252. van der Burg B, Ruterman GR, Blankenstein MA, DeLaat SW, van Zoelen EJJ. Mitogenic stimulation of human breast cancer cells in a growth factor-defined medium: synergistic action of insulin and estrogen. *J Cell Physiol* 1988;134:101.
253. Reddel RR, Murphy LC, Sutherland RL. Factors affecting the sensitivity of T-47D human breast cancer cells to tamoxifen. *Cancer Res* 1984;44:2398.
254. Strobl JS, Lippman ME. Prolonged retention of estradiol by human breast cancer cells in tissue culture. *Cancer Res* 1979;39:3319.
255. Freshney RI, Freshey I, eds. *Culture of animal cells. A manual of basic technique.* New York: Wiley-Liss, 1991.
256. Hayflick L. *Tissue culture.* New York: Academic, 1973:220.
257. *Methods in enzymology LVIII: cell culture methods.* New York: Academic, 1979.